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# Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique

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**BACKGROUND: Standard sperm characteristics are poor predictors of the outcome of IVF treatments. On the contrary, sperm genome quality has been emphasized for several years as playing a major role in early embryogenesis, thus in the success of IVF attempt. METHODS: Sperm DNA fragmentation from a selected group of 104 couples undergoing assisted reproductive techniques (ART) (IVF: $n = 50$; and ICSI: $n = 54$) was measured by TUNEL assay and correlated with semen and ART outcomes. RESULTS: A negative correlation was found between sperm characteristics and the proportion of sperm showing DNA fragmentation. For fragmentation >10%, a significant decrease of the fertilization rate was observed. No correlation was found between sperm DNA fragmentation and embryo quality. A high proportion of sperm with fragmented DNA was a pejorative factor to obtain pregnancies when ICSI was performed, but there was no relationship when conventional IVF was performed. CONCLUSIONS: The proportion of sperm with DNA fragmentation appears to be potentially useful as a predictor of ICSI outcome, whereas embryo quality based on morphological criteria, appeared unaffected by DNA fragmentation.**

*Key words:* ART/DNA fragmentation/embryo quality/human sperm

## Introduction

Assisted reproductive techniques (ART) such as conventional IVF, and especially ICSI, allow couples whose sperm characteristics are impaired to obtain a pregnancy, whereas a few years ago, these couples would have had to use sperm donation in order to obtain their child. One can nevertheless wonder about the capacity of poor quality sperm samples to generate embryos having normal capacities of development. Among the factors involved in the failure of obtaining embryos and/or pregnancies, the impaired sperm genome is frequently incriminated (Ahmadi and Ng, 1999a; Filatov *et al*., 1999; Larson *et al*., 2000). Several techniques of investigations are proposed in order to study these anomalies. Those which are currently used are the TUNEL technique which allows the evaluation of the sperm DNA fragmentation (Ahmadi and Ng, 1999b; Barroso *et al*., 2000), the Comet technique which represents another way of evaluating the DNA integrity (Chan *et al*., 2001; Morris *et al*., 2002), and DNA staining by acridine orange which differentiates between single and double stranded DNA based on their respective colours under fluorescence, and thus allows the degree of DNA denaturation to be evaluated (Larson *et al*., 2000). The investigation of DNA integrity by TUNEL gave similar results to that using sperm DNA staining with acridine orange, where the signal was quantified by flow-cytometry (Zini *et al*., 2001). In this study, we have analysed sperm DNA fragmentation in candidates for IVF or ICSI in order to establish the relationships between the proportion of sperm with fragmented DNA and various factors: quality of the embryos obtained, embryo development and more generally the rate of ongoing pregnancies.

## Materials and methods

### *Patients*

The prospective study concerned all the cycles of IVF/ICSI performed during a given period (4 months) within the Department of Medicine of the Reproduction in Edouard Herriot Hospital, Lyon, France: 104 cycles were included, being divided into 50 cycles of IVF, and 54 cycles of ICSI. ART procedures involving cryopreserved sperm samples or testicular extracted sperm, were excluded from this study. Only IVF/ICSI procedures with ejaculated sperm were included in this study.

### *Ovarian stimulation*

After three weeks of desensitization by GnRH analogues (Decapeptyl®; Ipsen), ovarian stimulation was achieved by recombinant FSH (Gonal-F®; Serono, or Puregon®; Organon), and monitored

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**Table I.** Overall data concerning ART procedures

| | IVF | | ICSI | |
|---|---|---|---|---|
| | Mean ± SD | Sample size | Mean ± SD | Sample size |
| Total oocytes | 11.4 ± 7.8 | 580 | 11.7 ± 4.8 | 618 |
| Mature oocytes | 9.2 ± 6.1 | 470 | 9.6 ± 4.3 | 510 |
| Total embryos | 7.8 ± 5.4 | 397 | 7.4 ± 3.5 | 391 |
| Normal embryos | 7.0 ±4.8 | 357 | 7.0 ± 3.4 | 372 |
| Transferred embryos | 2.2 ± 0.8 | 111 | 2.2 ± 0.7 | 115 |
| Cryopreserved embryos | 2.7 ± 3.9 | 89 | 1.3 ± 1.9 | 56 |
| Grade A | 2.1 ± 2.7 | 105 | 2.2 ± 2.1 | 119 |
| Grade B | 1.4 ± 2.2 | 72 | 1.2 ± 1.5 | 61 |
| Grade C | 0.5 ± 0.9 | 27 | 0.6 ± 0.9 | 33.0 |
| Grade D | 3.0 ± 2.8 | 153 | 3.1 ± 2.3 | 164 |

SD = standard deviation.

by endovaginal echography and plasma estradiol. When the follicles reached the desired diameter, 36 h before oocyte retrieval, 10 000 IU of hCG (Organon) was administered. The oocyte retrieval was carried out under general anaesthesia by a vaginal ultrasonographic-guided aspiration.

### *Sperm preparation for ART*

The sperm were prepared using a discontinuous PureSperm gradient (Nicadon, Gothemberg, Sweden). The gradient consisted of 3 layers of 1 ml of PureSperm: 90, 70 and 50%. On the 50% layer was deposited 1 ml of semen. The gradient was then centrifuged at 300 *g* for 20 min. After centrifugation, the 90% layer was collected and washed with 5 ml of Ferticult Flushing (FertiPro N.V., Beernen, Belgium) at 600 *g* for 10 min. The pellet was then resuspended in IVF Medium (Scandinavian IVF, Gothenburg, Sweden) for IVF or in HEPES IVF medium for ICSI.

### *Embryos*

At 16–18 h after insemination or microinjection, the oocytes were assessed for fertilization (two pronuclei (PN) stage). Then 48 h after oocyte retrieval, the embryos were classified according to their morphology. Classification was as follows: grade A: no fragmentation and four regular cells; grade B: <25% fragmentation; grade C: between 25 and 50% fragmentation and grade D: >50% fragmentation (Ebner *et al*., 2001). The transfer of the embryos took place either at 48 h, or at 72 h, or at the blastocyst stage. When the transfer was carried out at 48 or 72 h, the supernumerary embryos were cryopreserved if their morphological states allowed it (grade A or B). If they were not cryopreserved, they were cultivated in sequential medium until the blastocyst stage was reached, and if one or more good quality blastocysts were obtained, those were then cryopreserved. When a transfer at the blastocyst stage was programmed, embryos were cultivated in sequential medium: P-1 Medium (Irvine Scientific, Santa Ana, CA, USA) for the two first days, Blastocyst Medium (Irvine Scientific) for the last days of culture. After the transfer, the remaining good morphology blastocysts were cryopreserved. A clinical pregnancy was assessed by the succession of 3 positive plasma βhCG and ultrasound detection of a fetal heartbeat.

### *Semen sample preparation for DNA fragmentation study*

The detection of cells with fragmented DNA was performed on the spare sperm suspension that was used for ART procedure (the volume was between 50 and 60 µl of selected motile sperm suspension). All patients had given previously their informed consent for the study. The cells were spread out over sialinized slides. Cell fixation was carried out by a methanol/acetic acid mixture (3 volumes/1 volume) for 20 min. The cells were permeabilized with phosphate buffer saline (PBS) with 1% of Triton X100 (Sigma). Cells with fragmented DNA were revealed by TUNEL Technique by use of the 'Apoptag plus' Kit (Oncor, Illkirch, France). The cells were all first neutralized with the 'balanced buffer'. Then, sperm cells were incubated in a moist chamber at 37°C, for 1 h, with the Terminal d Transferase (TdT) solution in order to allow DNA elongation. The elongation was revealed by incubation of the cells with anti-digoxigenin antibody coupled to peroxidase, during 30 min in a dark moist chamber. The peroxidase was revealed with DiAminoBenzidine (DAB). Counterstaining of the sperm nucleus was performed with Harris' haematoxylin. A positive control was done on positive slides supplied in the Oncor Kit. The cells were observed under a microscope (Zeiss, Oberkochen, Germany) equipped with ×100 oil-objective. The sperm with fragmented DNA had their nuclei stained in brown, whereas the other cells nuclei were blue-grey. On each slide ~500 cells were counted, the percentage of sperm with fragmented DNA were thus determined.

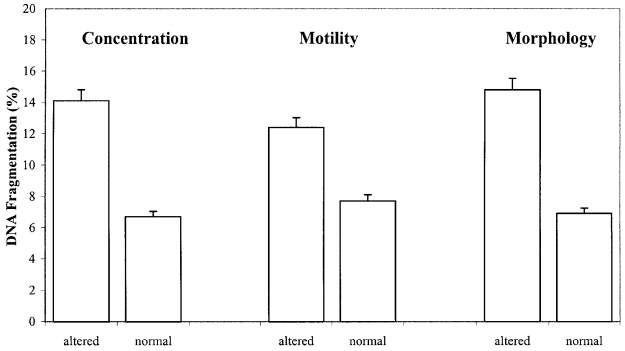


**Figure 1.** DNA fragmentation according to the sperm characteristics. The impaired concentration include case of oligospermia (there was no case of polyspermia).

### *Statistical evaluation*

Statistical analysis was performed with SPSS for Windows software package version 10.1 (SPSS Inc., Chicago, IL, USA). The $\chi^2$-test was used to analyse the qualitative parameters. For the comparison of quantitative parameters, the Student's *t*-test and ANOVA with mixed factors (mixed linear model) were used. Spearman's correlation coefficients were calculated. Statistical differences were considered significant at $P < 0.05$, for some comparisons the power analysis was calculated when no significant differences were found to ensure that



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the lack of significance could not be attributed to low sample number. The construction of a receiver-operating characteristic (ROC) curve allowed us to determine the thresholds values of DNA fragmentation having a prognostic role.

## Results

For the women, the mean age was 33.4 ± 3.8 years. A tubal deterioration was found in 27.5% of the cases, a dysovulation in 29.8% of the cases. 27.3% of the women suffered from an endometriosis. For 2.9% of patients, a uterine anomaly was found.

For the men, the mean age was 35.5 ± 4.9 years. In 38.5% of the cases, the sperm used for the ART procedure presented anomalies according to World Health Organization (WHO) standards (World Health Organization, 1999). An oligospermia was found in 26% of the cases, an asthenospermia in 19.2% and a teratospermia in 15% of the cases. These anomalies were not always isolated.

The mean (± SD) quantity of FSH administered to the patients was of 2823 ± 1351 IU, the average time of stimulation was of 12.1 ± 1.6 days and the estradiol rate at the day of the puncture was of 2675 ± 1556 pg/ml. The results were summarized according to the ART procedure used (Table I).

### *DNA fragmentation and sperm characteristics*

A significant negative correlation was observed between DNA fragmentation and sperm concentration ($r = –0.44$, $P < 0.01$); between fragmentation and sperm motility ($r = –0.28$, $P < 0.05$) and between fragmentation and the percentage of atypical forms ($r = –0.36$, $P < 0.01$). The classification of sperm samples according to WHO standards, allowed us to highlight an increase in the proportion of sperm with DNA fragmentation in cases of impaired sperm characteristics (Figure 1).

### *DNA fragmentation and fertilization rate*

The fertilization rate did not seem to be influenced by sperm fragmentation whatever the technique used: $r = – 0.1$ (not significant) for IVF and $r = – 0.14$, (not significant) for ICSI. However, when DNA fragmentation was divided into two categories according to a threshold value of 10%, the fertilization rate was significantly higher for DNA fragmentation below 10% (84.1 versus 70.7%, $P < 0.05$) (Figure 2).

### *DNA fragmentation and embryo quality*

The total rate of good quality embryos was 48.6%. No relationship between fragmentation and embryo quality was found (Figure 3). The rate of good quality embryos was

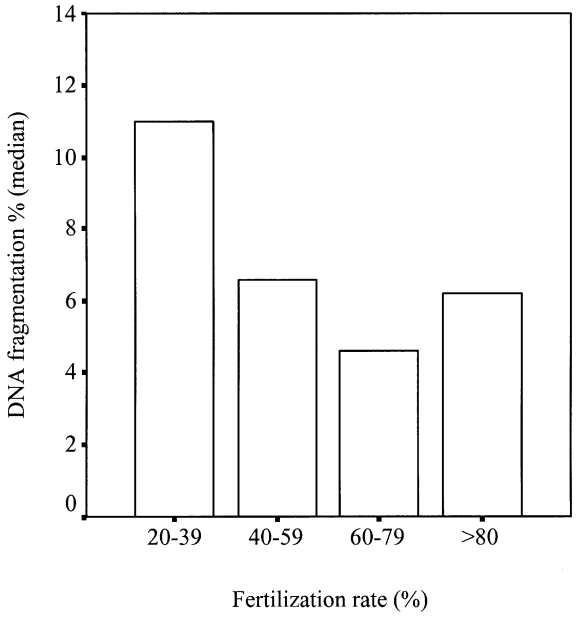


**Figure 2.** Sperm DNA fragmentation according to the fertilization rate.

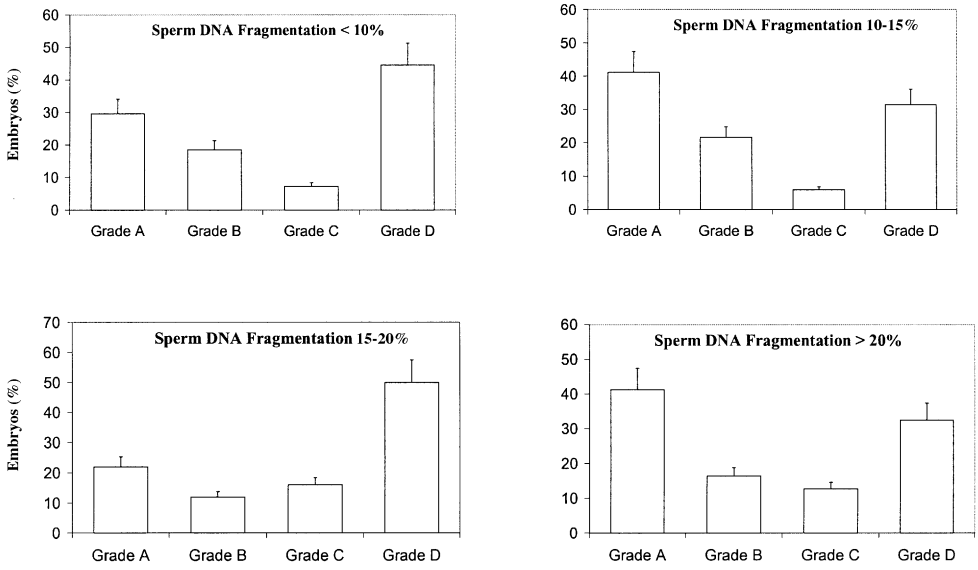


**Figure 3.** Embryo quality distribution according to sperm DNA fragmentation.

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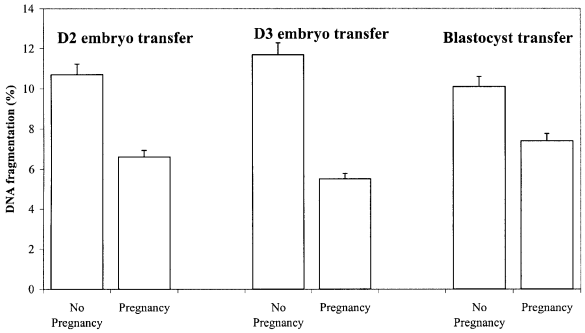


**Figure 4.** Sperm DNA fragmentation and issue of ART when ICSI was performed.

**Table II.** Relative Risk, sensitivity and specificity for obtaining a pregnancy according to different threshold values of the sperm DNA fragmentation

| DNA fragmentation | | | | |
|---|---|---|---|---|
| Threshold value | Pregnancy rate (%) | Relative risk | Sensitivity | Specificity |
| < 4% | 22.7% | 0.92 | 0.76 | 0.25 |
| > 4% | 21.4% | | | |
| < 15% | 22.8% | 0.61 | 0.15 | 0.9 |
| >15% | 15.4% | | | |
| < 18% | 23.2% | 0.37 | 0.14 | 0.95 |
| >18% | 10.0% | | | |
| < 20% | 23.8% | NA | 0.11 | 1.0 |
| > 20% | 0.0% | | | |

NA = not available.

independent of the proportion of sperm with DNA fragmentation: 45.7 ± 32.6% versus 43.9 ± 28.2% (not significant, with a power analysis of 6%), for fragmentation below and above 15% respectively. With this sample size the minimum detectable difference was 15%.

### *DNA fragmentation and embryo development*

When a blastocyst transfer was undertaken (25 cases), at least one blastocyst was obtained in 84% of the cases. No significant difference according to the ART procedure was found (87.5% in IVF and 81.8% in ICSI). The average percentage of embryos cleaved on D2 and reaching the blastocyst stage was 36.2 ± 25%. This percentage of blastocysts was independent of the ART procedure (37.7% in IVF and 34.2% in ICSI, not significant). When no blastocysts were obtained, DNA fragmentation was not significantly increased (8.3 versus 7.8%). However, when DNA fragmentation was higher than 10%, the percentage of embryos reaching the blastocyst stage was always lower than 50%, whereas this rate could reach 80% when DNA fragmentation was lower than 10%.

### *DNA fragmentation and pregnancy*

The pregnancy rate was influenced neither by the sperm characteristics, nor the type of ART procedure.

According to the ART procedure, when IVF was performed, DNA fragmentation was not statistically different whether a pregnancy was established or not. When ICSI was carried out, DNA fragmentation was significantly lower when a pregnancy was obtained ($P < 0.05$) irrespective of the age of the embryos at the time of the transfer (Figure 4).

We looked for a threshold value of DNA fragmentation that would allow us to predict the establishment or not of a pregnancy. Construction of a ROC curve showed that with a threshold of fragmentation not higher than 4% the sensitivity was 76% for a specificity of 25%. If the threshold was fixed at 15% or even at 18%, the specificity of the fragmentation factor was increased (90 and 95% respectively) but the sensitivity fell dramatically (15 and 14% respectively). The relative risks (RR) of obtaining a pregnancy were 0.61 and 0.37 respectively (Table II). No pregnancy was obtained when DNA fragmentation was higher than 20%. The area under the curve was 0.5.

## Discussion

Impairments of sperm characteristics were associated with an increase in the proportion of sperm with DNA fragmentation confirming the results of various recent studies (Sun *et al*., 1997; Lopes *et al*., 1998; Gandini *et al*., 2000; Irvine *et al*., 2000; Younglai *et al*., 2001). For a threshold value above 10%, we found a significant negative relationship between sperm DNA fragmentation and the fertilization rate, in disagreement with some authors (Tomlinson *et al*., 2001; Morris *et al*., 2002), but in agreement with others who found that sperm DNA fragmentation rate negatively correlated with fertilization rate in an ICSI programme (Lopes *et al*., 1998; Host *et al*., 2000). However it is interesting to note that in Lopes' work as in our study, sperm DNA fragmentation did not vary for fertilization rates >40%, but increased for fertilization rates of <40%. It is possible that if DNA fragmentation is low, the oocytes are able to repair the damaged sperm DNA (Sakkas *et al*., 1996; Ahmadi and Ng, 1999b), but these capacities are overloaded in cases of high level of sperm DNA fragmentation. Sakkas *et al*. (1996) postulated that damaged sperm DNA might contribute to failure of sperm DNA decondensation after ICSI, thus resulting in fertilization failure. On the other hand, Host *et al*. (2000) assumed that in ICSI the operator tries to select a motile, and as far as possible, a morphologically normal spermatozoon, so this cell has an improved chance of also having intact DNA. However, it can be argued that a spermatozoon can be considered as 'normal' and at the same time have damaged DNA (Lopes *et al*., 1998; Tomlinson *et al*., 2001).

Ahmadi and Ng (1999a) showed that high sperm DNA fragmentation did not impair fertilization but precluded the blastocyst formation. For example, oxidative damage to sperm DNA does not impede pronucleus formation after microinjection in hamster eggs (Twigg *et al*., 1998). This observation is rather logical since it is generally assumed that the first steps of development are under the dependence of the maternal transcripts, thus the paternal expression would normally start at the 6–8 cell stage; it must be reminded that in the majority of cases embryo transfers are performed at day 2 or day 3, i.e. before the paternal effect can be expressed. However, recent studies have shown that the paternal genome could play a role very early in the human oocyte development, as soon as the first cell cycle (Tesarik *et al*., 2002). This could explain the



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controversies about the impact of sperm DNA fragmentation on fertilization rate, since this 'early' effect is certainly less important than the alternative effect observed at the 6–8 cells stage; it is therefore a logical hypothesis that DNA fragmentation may indeed impair fertilization, but only for highly fragmented DNA as observed in the present study. Whereas there are still controversies about the impact of high sperm DNA fragmentation on the fertilization rate, there is a wider agreement concerning their negative effects on embryo development (Ahmadi and Ng, 1999a; Morris *et al*., 2002) and pregnancy rate (Ahmadi and Ng, 1999a; Host *et al*., 2000; Larson *et al*., 2000; Tomlinson *et al*., 2001).

In our study this deleterious effect of high sperm DNA fragmentation rate was significant in ICSI cycles, but not significant in IVF cycles, in disagreement with Tomsu *et al*. (2002) who used Comet assays for evaluating sperm DNA damage. This difference may be explained as follows: since most ICSI cycles are performed because of poor sperm characteristics (that is not the case in IVF), it is logical to find a higher rate of sperm cells with elevated DNA fragmentation in ICSI than in IVF cycles. Moreover, it is possible that IVF steps lead to a 'natural' selection of sperm: the selected fertilizing spermatozoon will be morphologically normal and highly motile, and supposed to have an intact DNA. This hypothesis is supported by recent studies, including the one of Van Dyk *et al*. (2000) who suggested that the human zona pellucida had the capacity to select against aneuploid sperm. It was shown by several authors that in a poor quality sperm population (according to classical criteria), DNA damage is found at a high level (Sun *et al*., 1997; Lopes *et al*., 1998; Gandini *et al*., 2000; Irvine *et al*., 2000; Younglai *et al*., 2001); however, such sperm with DNA damage would have very few chances of fertilizing the oocyte if they were used in an IVF procedure. The problem is different in ICSI, where the choice of the spermatozoon to be injected is made according to very rough criteria, and in case of very poor sperm characteristics, it is even not possible to select one normal motile sperm so the risk of injecting one spermatozoon with impaired DNA is high.

These observations can have a number of applications in certain situations. For example, it has been shown that testicular extracted sperm have lower DNA fragmentation than sperm retrieved from the epididymis (Steele *et al*., 1999). So if the choice between testicular and epididymal sperm is possible—in cases of obstructive azoospermia—testicular extracted sperm should be preferred to epididymal sperm for ICSI in order to obtain embryos with a good development potential.

Considering the overall data (ICSI and IVF cycles), we showed an important drop in the relative 'risk' of pregnancy as the proportion of sperm with DNA fragmentation reached higher than 18%. The specificity values reached 90 and 95% for fragmentation proportions of 15 and 18% respectively. With the aim of defining the best strategy for couples, it seems more important to look for high specificity rather than a high sensitivity: thus the observation of no pregnancy occurring in our study for fragmentation higher than 30%, can constitute a strong argument to counsel some couples, who have already made several attempts with systematic embryo transfers, but without pregnancy, and who wonder about the usefulness of beginning one more IVF treatment cycle. Nevertheless, the sample size does not allow the drawing of definitive conclusions, since the area under the ROC curve is 0.5, which represents a minimal acceptable value.

For those patients exhibiting a high proportion of sperm with fragmented DNA (≥20%), a solution would consist in the elimination—or at least the reduction—of the cells with damaged DNA from the sperm population prepared for ART procedures. The cells could be sorted with flow cytometry. This is technically possible, but the problem is to be sure that the technique by itself, which necessitates DNA labelling and laser illumination, has no deleterious effects on sperm DNA. Donnelly *et al*. (2000) suggested that Percoll density gradient centrifugation was useful in isolating a sub-population of sperm exhibiting a lower proportion of fragmented DNA than in the original semen, using both the Comet and the TUNEL assays. However, the values measured in our study were already obtained after density gradient selection, and one can expect that the percentages of DNA fragmented sperm in the original ejaculate were much higher. Moreover, some doubts may be raised about the DNA quality of the cells that are not labelled by the Comet or TUNEL assays, but may be in a pre-fragmentation state. For example, if the high level of DNA fragmentation is due to the presence of reactive oxygen species or to the activation of apoptotic factors, it is doubtful that the DNA in these negative cells will remain undamaged.

In conclusion, our data indicate that the proportion of sperm with DNA fragmentation influences the fertilization rate for a threshold value above 10%, and an implantation rate of ICSI-derived embryos. Since no pregnancy was obtained if >20% of selected sperm were TUNEL positive, this factor may have a good predictive value in cases of successive failures of implantation for apparently good quality embryos.

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