



Research article

Density gradient capacitation is the most suitable method to improve fertilization and to reduce DNA fragmentation positive spermatozoa of infertile men

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ABSTRACT

Objective: To investigate the relationship between DNA fragmentation by TUNEL method and seminogram parameters from fresh and capacitated samples for swim-up and gradient methods, to verify the best method for improving the outcome of in vitro fertilization.

Materials and methods: Samples were obtained from 100 volunteers after 4-5 days of sexual abstinence. Classical semen parameters were analyzed by computer-aided sperm analysis (CASA) according to WHO classification criteria. Semen was washed twice in RPMI medium and every sample was divided into 3 parts. Two parts were used to capacitate the semen by swim-up and gradient methods, and the other was taken as fresh sample. Analysis of apoptosis and vitality were carried out by TUNEL, and by acridine orange and ethidium bromide methods, respectively on both fresh and capacitated samples.

Results: Our findings show a negative correlation between TUNEL-positive spermatozoa and the efficiency of pregnancy as well as concentration and mobility. To recover mobile cells by capacitation methods, gradient was more efficient than swim-up as well as removing apoptotic and necrotic cells, hence, enriching the quality of semen samples.

Conclusion: DNA fragmentation is a useful tool to differentiate between fertile and infertile men, thus, DNA fragmentation analysis should be part of routine semen analysis for patients suffering infertility.

Key words: Seminogram parameters, capacitation methods, TUNEL, spermatozoa, apoptosis.

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Introduction

Semen quality is usually based on the seminogram parameters standardized by World Health Organization (WHO) such as motility, concentration and morphology. Unfortunately, analysis of these parameters is subjective and prone to significant inter- and intraobserver variability [1]. Additionally, these parameters are also sensitive to possible contacts with toxic agents [2, 3] and environmental exposures, that could produce alterations in semen characteristics and spermatozoid genome [3].

There are some intrinsic spermatozoid abnormalities that are not detected with usual sperm analysis but have important implications when the semen is used for assisted reproductive technologies. Spermatozoa with nuclear abnormalities have been associated with abnormal fertilization and embryo fragmentation [4, 5]. It is known that sterile male patients have some defects in both structure and function of spermatozoa [6, 7].

The chromatin of a mature spermatozoid is highly condensed due to the replacement of histones with protamines during the spermiogenesis. This definitive packing of the chromatin is essential for reorganizing the genomic material and expressing appropriate genes in the development of embryo [8]. Although this packing is essential for expression of fertility, it is not always foolproof. These errors can be detected by acridine orange or chromomycin A3 stains. The amount of damaged DNA transmission that exceeds the repair capacity of oocyte may have serious consequences compromising possible gestations [9]. The previous data call into a question whether spermatic capacitation methods for assisted reproductive techniques are effective or, on the contrary, spermatozoa with damaged DNA can overcome this selection and be used for fertilization.

In vitro fertilization (IVF) clinics perform two techniques to isolate viable spermatozoa: swim-up, which uses swimming ability of spermatozoa to select those ones with the best motility, or discontinuous gradient, which uses



centrifugation to separate the motile and morphologically better spermatozoa [10, 11].

The number of spermatozoa with normal chromatin condensation decreases significantly following swim-up with respect to fresh samples [12]. Subsequent studies showed that swim-up technique could improve seminal parameters related to the structure of the chromatin [13] without induction of DNA damage [14]. As a passive selection method this procedure is more physiological that involves minimal manipulation of samples. Selection of motile spermatozoa depends on the percentage of motile spermatozoa in the initial sample, the type of motility, interface surface, incubation time [15], and the culture medium composition [16]. Other studies have contributed that gradient-selected motility spermatozoa have a higher membrane potential, less fragmented nuclear DNA, and lesser reactive oxygen species compared to the initial sample [17, 18]. Usually a 45%-90% or three-layered discontinuous density gradients are used [19] with colloidal silica particles covered by PVP (polyvinylpyrrolidone). Although first used in 80s, gradient method was standardized with the Percoll withdrawal after 1996. Only the 90% density gradient may be used in artificial insemination with normal semen parameters [20].

Other studies have compared the effects of the swim-up and gradient methods on the spermatid integrity. Sakkas *et al.*, [21] reported that the gradient technique resulted in a significant decrease in the percentage of spermatozoa with damaged DNA. However, similar improvement was not observed with the swim-up technique. Contrary to this opinion, Zini *et al.*, [22] published that, compared to initial semen sample, a significantly reduced percentage of spermatozoa with denatured DNA was obtained with swim-up, but not with density gradient. Obviously, there is no agreement about the technique of choice.

The recent studies show an increased interest in detection of damage on spermatid DNA integrity using the analysis of apoptotic markers [23-25].

The aim of this study was to compare the viability and the DNA fragmentation between swim-up and gradient

capacitation methods, to verify the sperm characteristics, such as low sperm concentration, motility or normal forms that were associated with the proportion of spermatozoa with DNA fragmentation, and finally, to correlate DNA fragmentation with the sperm quality for fertilization.

Materials and methods

Patients

Samples were obtained from 100 volunteers between 25 and 45 years old (25 patients for each group), at the Extremadura Center of Human Assisted Reproduction (Badajoz, Spain). Each subject was ascertained to be in good health by means of their medical history and clinical examination including routine laboratory tests and screening. The subjects were all non-smokers, not using any medication, and abstained from alcohol. Informed consent was obtained from all participants.

The study was approved by the Institutional Review Board of the University of Extremadura and by the Ethics Committee of the Infantile Hospital (Badajoz, Spain) and was conducted in accordance with the Declaration of Helsinki. No financial conflicts of interest exist with any commercial entity whose products are described, reviewed, evaluated, or compared in this study.

Semen collection and analysis

Samples were collected by masturbation after 4-5 days of sexual abstinence, and were allowed to liquefy at 37°C for 30 min. Semen was washed twice in RPMI medium (250 g, 10 min); the supernatant was discarded, and the sperm pellet was resuspended in Na-HEPES solution containing (in mM): NaCl 140, KCl 4.7, CaCl₂ 1.2, MgCl₂ 1.1, glucose 10, and HEPES 10, with pH 7.4. The classical semen parameters of spermatozoa concentration, motility, and morphology were examined according to WHO criteria [26], which are shown in the last column of **Table 1**. Computer-aided sperm analysis (CASA) was used for evaluation of samples.

Table 1. Semen parameters in fresh ejaculates from normospermic and infertile patients.

| | Normospermic (mean ± SD) | Oligospermic (mean ± SD) | Asthenospermic (mean ± SD) | Teratospermic (mean ± SD) | WHO criteria (range) |
|---|-----------------------------|-----------------------------|-------------------------------|------------------------------|-------------------------|
| Volume (ml) | 4.08 ± 1.49 | 3.9 ± 1.52 | 3.35 ± 1.69 | 4.5 ± 1.26 | - |
| Concentration (×10 ⁶ /ml) | 42.61 ± 16.84 | 6.47 ± 2.0* | 43.12 ± 14.7 | 39.87 ± 8.13 | 57.5-160 |
| Total Concentration (×10 ⁶) | 142.04 ± 38.05 | 23.77 ± 4.19 | 83.35 ± 13.0 | 152.5 ± 21.91 | - |
| Motility A + B (%) | 54.6 ± 4.66 | 48.35 ± 6.57 | 36.73 ± 6.19* | 55.5 ± 6.0 | 40-70 |
| Round cells (%) | 2.15 ± 1.06 | 2.03 ± 1.22 | 3.6 ± 1.76 | 2.8 ± 1.0 | 0.5-2 |
| Morphology (% normal) | 19.0 ± 2.1 | 17.2 ± 2.5 | 16.8 ± 1.2 | 10.1 ± 0.9* | 14-21 |

SD, Standard deviation

* p<0.05 compared with WHO values

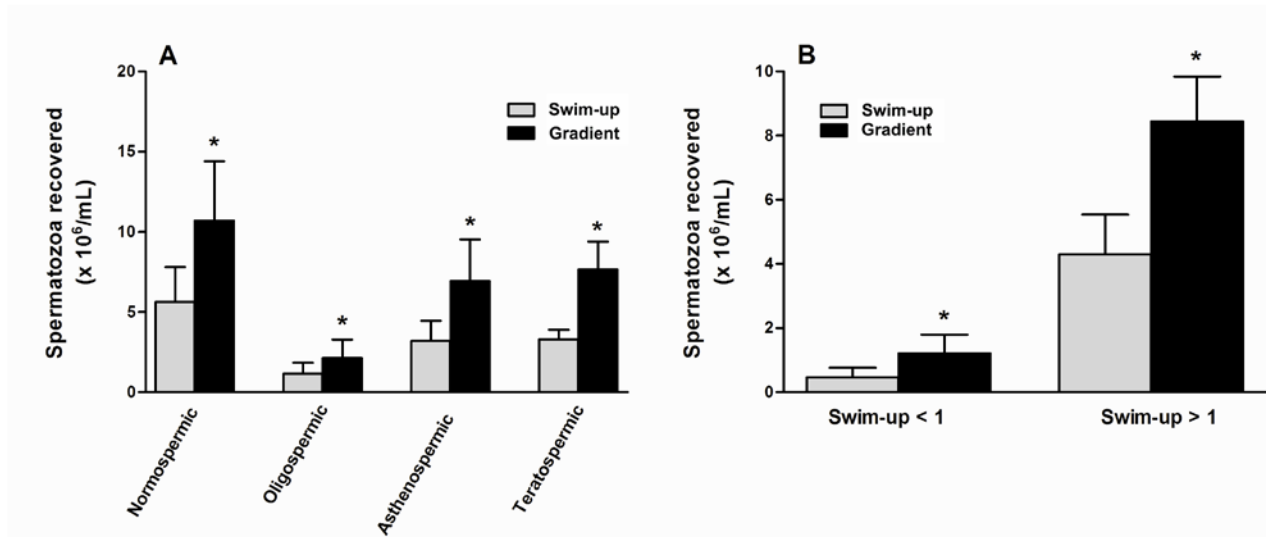


Figure 1. The effect of separation procedure on the number of spermatozoa recovered. **A.** Number of motile spermatozoa recovered in both fresh semen and semen after normal swim-up (swim-up) or 90% density gradient (gradient), which was obtained from healthy donors and infertile patients. **B.** Motile spermatozoa recovered from semen after normal swim-up or 90% density gradient when spermatozoa recovered by swim-up technique was lower (swim-up < 1) or higher (swim-up > 1) than 10⁶ cells/ml. Values are expressed as means \pm SD of fifteen separate determinations.

* $p < 0.05$ compared with swim-up group.

Capacitation methods

Samples were washed 1:1 with PBS and were centrifugated at 250 g for 15 min. The pellet was resuspended in 1.3 ml culture medium. 300 μ l were taken from the sample for apoptosis determination through TUNEL assay and the rest was divided into two equal parts. The first one was used to carry out the capacitation by swim-up. The sample was resuspended in culture medium with no seminal plasma, and then it was centrifugated at 250 g, 15 min. The supernatant was removed and 0.5 ml of fresh medium was carefully added on the pellet. The tubes were tilted 45° and the concentration of mobile spermatozoa which migrated to the pellet was measured after incubation for 45 min at 37°C.

The second part of the sample was used for gradient capacitation; 0.5 ml of resuspended pellet was gently placed over 1 ml of 90% density column. After centrifugation at 250 g for 15 min, the supernatant was removed. The pellet was resuspended in 0.5 ml of fresh medium and a mobile spermatozoa count was performed after incubation for 45 min at 37°C. Both counts were classified as motile spermatozoa recovered. Finally, vitality assessment and TUNEL of capacitated samples by both methods were carried out.

Vitality dying

The samples were stained with acridine orange and ethidium bromide in order to identify viable, capacitated spermatozoa. For staining 50 μ l and 10 μ l each of acridine orange (Sigma; 100 μ g/ml in normal saline, pH 7.4) and ethidium bromide (Bio-Rad; 100 μ g/ml in normal saline, pH 7.4) were added separately [27]. After 30 minutes of incubation at room temperature with the dyes, 20 μ l were

placed on a glass slide. All samples were examined using the same microscope. Orange spermatozoa were considered as dead and green spermatozoa as alive.

TUNEL assay

DNA damage can be detected by TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay, as previously described [28, 29]. DNA fragmentation was measured using the *in situ* cell death detection kit fluorescein (Roche), which used fluorescein-dUDP as label according to the manufacturer's instructions. TUNEL assay is a well-established method for the detection of DNA fragmentation, a relatively late apoptotic marker [30]. This technique has been widely used to determine DNA damage in human spermatozoa [28, 29].

Semen samples were washed, the supernatant was discarded and the cell pellet was finally resuspended in Na-HEPES solution. Each sample was divided in three fractions. One fraction contained resuspended fresh spermatozoa, and the other two fractions were capacitated by swim-up or gradient methods.

Three slides were air-dried for 24 hours and then washed in PBS. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 (Sigma). The cells were incubated in dark at 37°C for 1 hour in TUNEL reaction mixture, containing 50 μ l of the mixture of terminal deoxynucleotidyl transferase and dUDP. At least 100 cells were randomly analyzed per slide in five fields. Spermatozoa were first identified in the field using phase contrast microscopy and then analyzed using fluorescence microscopy. Each cell was assigned as apoptotic (intense green nuclear fluorescence) or normal (no fluorescence).

Statistical analysis

Coefficient of determination (r^2) was calculated to compare the sperm concentration, forward motility and pregnancy rate, with the different percentage of TUNEL-positive cells. Means were compared by analysis of variance (ANOVA) test. Tukey’s test was used for *post hoc* comparisons. Data were expressed as mean \pm standard deviation (SD). Statistical procedures were carried out by GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

A comparison of the efficiency of the swim-up and gradient separation procedures is shown in **Figure 1A**. The number of recovered sperm cells was increased ($p < 0.05$) after sperm separation with swim-up or gradient procedure in normospermic as well as oligospermic, asthenospermic and teratospermic patients. Additionally, there were also differences between the procedures that the number of sperm cells recovered with the gradient method was greater than in the swim-up method in all groups analyzed ($p < 0.05$). Moreover, gradient method increased the number of spermatozoa recovered to 1.22 ± 0.57 million (2.62 fold) of cells per milliliter ($p < 0.05$) while the number of cells recovered by the swim-up method was lower than one million of cells per milliliter (**Figure 1B**). This finding is clinically important because the values of recovered spermatozoa over one million of cells per milliliter are enough to ensure a successful in vitro fertilization (IVF) procedure. Even in cases with swim-up method yield higher than one million of cells per milliliter, gradient method yield 1.96 times more cells ($p < 0.05$) (**Figure 1B**).

As shown in **Figure 2A**, fresh spermatozoa from healthy donors and infertile patients exhibited DNA fragmentation, indicating that spermatozoa might be undergoing late stages of apoptosis. However, fresh sperm cells from all groups of infertile patients showed higher ($p < 0.05$) levels of DNA fragmentation compared with normospermic donors. Additionally, we checked the efficiency in removing apoptotic cells from the fresh samples by both using the swim-up and gradient methods (**Figure 2A**). After swim-up method percentage of TUNEL-positive cells was significantly reduced in infertile patients ($p < 0.05$). However, after gradient method percentage of apoptotic cells was significantly decreased in both healthy donors and infertile patients ($p < 0.05$). Moreover, gradient method was more effective in removing apoptotic cells in both normospermic and asthenospermic men ($p < 0.05$).

We also tested the efficiency of both methods in removing necrotic cells from the fresh samples (**Figure 2B**). In this respect, both methods were also effective in removing necrotic cells. After swim-up procedure, the percentage of necrotic cells was reduced ($p < 0.05$) in all groups in oligospermic patients. After the gradient method, the percentage of necrotic cells was reduced ($p < 0.05$) in all groups analyzed. Also, gradient method was more effective than the swim-up method in removing necrotic cells in oligospermic patients ($p < 0.05$).

To analyze the relationships between the percentage of TUNEL-positive cells and different seminal parameters,

Table 2. Semen parameters in fresh ejaculates from donors and infertile patients according to World Health Organization (WHO).

| | TUNEL < 15% | TUNEL 15-30% | TUNEL > 30% |
|------------------------------------|--------------------|-------------------|------------------|
| Concentration ($\times 10^6$ /ml) | 36.25 \pm 8.56*† | 19.44 \pm 8.45† | 12.5 \pm 2.88 |
| Motility A + B (%) | 52.26 \pm 5.87*† | 41.31 \pm 9.39† | 30.33 \pm 7.57 |

* $p < 0.05$ regarding TUNEL 15-30% values

† $p < 0.05$ regarding TUNEL > 30% values

Table 3. Semen parameters in fresh ejaculates from donors and infertile patients according to World Health Organization (WHO).

| | n | Pregnancy (cases) | PR (%) |
|--------------|----|-------------------|----------|
| TUNEL < 15% | 21 | 10 | 47.62 *† |
| TUNEL 15-30% | 18 | 6 | 33.33 † |
| TUNEL > 30% | 10 | 0 | 0.0 |

* $p < 0.05$ regarding TUNEL 15-30% values

† $p < 0.05$ regarding TUNEL > 30% values

correlations between the sperm concentration and the forward motility and the different percentage of TUNEL-positive cells were analyzed (**Table 2**). The sperm concentration and the forward motility were negatively correlated ($r^2 = 0.882$ and 0.987 , respectively) with the percentage of TUNEL-positive cells. These findings indicate that the increase in the sperm quality was the result of removal of defective or apoptotic spermatozoa. Moreover, there is no correlation between morphological alterations (in sperm head, midpiece, and tail) in spermatozoa and the percentage of TUNEL-positive cells (unpublished data).

Finally, we further investigate the relationships between the percentage of TUNEL-positive cells and the sperm quality. In this way, we analyzed the IVF pregnancy rate (PR) depending on different percentages of TUNEL-positive cells in fresh ejaculates (**Table 3**). The PR reached its peak (47.62%) when the percentage of TUNEL-positive cells was lower than 15%. Additionally, in the first group (TUNEL < 15%), 77% were normospermic men and 100% exhibited values of DNA fragmentation lower than 11% (unpublished data). Furthermore, no pregnancy was recorded when the TUNEL-positive cells were higher than 30%. Interestingly, we have also found a negative correlation between PR and percentage of TUNEL-positive cells ($r^2 = 0.985$), indicating that the sperm quality is important for a successful fertilization, and a positive correlation between age and percentage of TUNEL-positive cells ($r^2 = 0.978$), indicating a deterioration in semen quality with age which reduced the probabilities of fertilization (unpublished data).

Discussion

Previous studies on comparisons between swim-up and gradient separation methods reported controversial results. However, it is generally accepted that gradient method gives better sperm concentration than swim-up [31-34], except for Branderis and Manuel [35]. In accordance with previous studies, our results showed that the gradient method is superior for spermatozoa selection due to higher sperm quality obtained with this technique.

Sperm DNA integrity is essential for the accurate transmission of genetic information. The clinical significance of this assessment lies in its association with not only natural conception rates, but also the success of assisted reproduction technology (ART). Sperm DNA damage can be measured directly by TUNEL assay, which has been widely used to determine human sperm DNA damage [28, 29]. It has been shown that the fraction of abnormal sperm detected by TUNEL is higher in infertile men. This is also compatible with the previous findings obtained by using the sperm chromatin structure assay (SCSA) method [36-39]. Likewise, our findings demonstrated that impairments of sperm characteristics, such as low sperm concentration, motility or normal forms, were associated with an increase in the proportion of sperm with DNA fragmentation confirming the results of recent studies [4, 14, 40-43]. Additionally, a significant negative correlation between DNA fragmentation and sperm concentration, and between fragmentation and sperm motility is confirming the results reported by Oosterhuis *et al.* [44], and Benchaib *et al.* [42]. Although, Benchaib *et al.* also reported a negative correlation between the fragmentation and the percentage of atypical forms, we failed to demonstrate similar correlation among these two parameters.

Several studies have shown that sperm DNA quality had robust power to predict IVF outcome [40, 45-47]. It has been shown that high load of DNA damage was predictive for the embryonic development failure after intracytoplasmic sperm injection (ICSI) [48]. Similarly, using the Single Cell Gel Electrophoresis assay (comet assay), Tomsu *et al.* [49] noted that the comet head and tail DNA parameters could be considered potentially useful predictors of embryo quality and IVF outcomes, especially in couples with unexplained subfertility. We found a significant negative relationship between sperm DNA fragmentation and the fertilization rate. In this respect, our results were in accordance with previous reports with traditional IVF procedures as well as with ICSI program studies [4, 40, 50, 51] while opposite findings were also reported [49] these results are compatible with other studies that reported negative correlation between sperm DNA fragmentation and ICSI fertilization rates [4, 40, 50, 51] or with IVF procedure [52]. It seems to be semen samples with high rates of DNA breaks as assessed by the SCSA [53] or TUNEL [50] methods are more likely to result in pregnancy in ICSI procedures than traditional IVF. Using these findings, a large-scale conclusion could be reached, but randomized studies are needed to determine the effects of sperm DNA damage on the outcome of ART in different clinical settings.

Benchaib *et al.* [40] showed an important, relative "risk" of pregnancy when the proportion of sperm with DNA

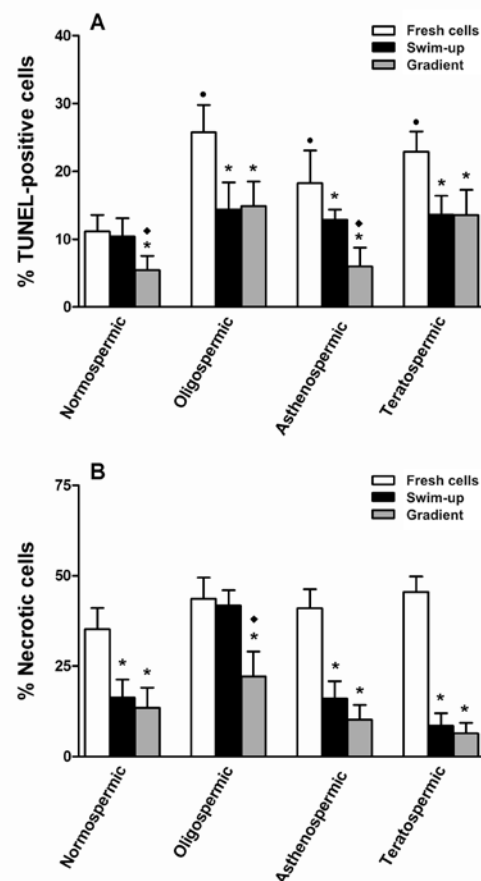


Figure 2. Effect of separation procedure on the percentage of both apoptotic and necrotic cells. DNA fragmentation (assessed by TUNEL assay) (A) and the percentage (%) of necrotic cells (B) were determined as described in materials and methods. In both fresh semen (fresh cells) and semen after normal swim-up (swim-up) or 90% density gradient (gradient), which was obtained from healthy donors and infertile men. Values are expressed as means \pm SD of twelve separate determinations. * $p < 0.05$ compared with values obtained in fresh cells group. ♦ $p < 0.05$ compared with values obtained in swim-up group. • $p < 0.05$ compared with values obtained in normospermic donors.

fragmentation exceeded 18%. In fact, our findings showed a significant reduction of IVF success when DNA damage index was higher than 15%. Furthermore, we found that men with high levels of DNA fragmentation (>30 % TUNEL-positive cells) failed to initiate an ongoing pregnancy, in accordance with the results obtained by Virro *et al.* [54]. Then, 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. Donnelly *et al.* [17] suggested that density gradient centrifugation was useful in isolating a sub-population of sperm exhibiting a lower proportion of fragmented DNA than the original semen, using both the comet and the TUNEL assays, which is in accordance with the findings with our study. However, some doubts may be

raised about the DNA quality of the cells that are not labeled by the comet or TUNEL assays, but may be in a prefragmentation 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 summary, our findings demonstrate that gradient method rendered a higher number of spermatozoa recovered and showed to be more effective in removing defective cells from the seminal plasma, compared with the swim-up procedure. Moreover, our data indicate that the cut-off of sperm DNA fragmentation is a useful tool to differentiate between fertile and infertile men, as applicable to the in vivo situation. Further research will be necessary to see if techniques can be devised to identify and select sperm with undamaged DNA for IVF, or to remove sperm with damaged DNA from sperm samples, to enable the pregnancy outcome after IVF to be improved. Therefore, we suggest that the tests analyzing sperm DNA quality should be a part of the routine semen analysis for infertile patients regardless of the choice of treatment.

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