BIOLOGY OF REPRODUCTION 56, 602–607 (1997)

Detection of Deoxyribonucleic Acid Fragmentation in Human Sperm: Correlation with Fertilization In Vitro[1]

Jian-Guo Sun, Andrea Jurisicova, and Robert F. Casper[2]

Division of Reproductive Sciences, Department of Obstetrics and Gynecology, University of Toronto, and the Toronto Hospital Research Institute, Toronto, Ontario, Canada M5G 2C4

ABSTRACT

The objective of this study was to determine the incidence of DNA fragmentation in human sperm, and to correlate any detected DNA damage with semen analysis parameters and fertilization rates in in vitro fertilization (IVF). A total of 298 semen samples were collected from men in the infertility program at The Toronto Hospital. For each sample, the percentage of sperm with DNA fragmentation was determined using the method of terminal deoxynucleotidyl transferase-mediated dUTP-biotin end-labeling (TUNEL) and fluorescence-activated cell sorting. The percentage of sperm with fragmented DNA was less than 4% in the majority of samples but ranged from 5% to 40% in approximately 27% of the samples. A negative correlation was found between the percentage of DNA fragmentation and the motility, morphology, and concentration of the ejaculated sperm. In 143 IVF samples, a significant negative association was also found between the percentage of sperm with DNA fragmentation and fertilization rate ($p = 0.008$) and embryo cleavage rate ($p = 0.01$). In addition, 35 men who smoked demonstrated an increased percentage of sperm with fragmented DNA (4.7 ± 1.2%) as compared to 78 nonsmokers (1.1 ± 0.2%; $p = 0.01$).

These results demonstrate a negative association between semen analysis parameters and sperm with fragmented DNA. Since extremely poor semen samples are the indication for intracytoplasmic sperm injection, there is a high likelihood that sperm with fragmented DNA may be selected by chance and used for oocyte injection, resulting in poor fertilization and/or cleavage rates.

INTRODUCTION

It is well established that sperm quality plays a crucial role in human reproduction. Routine semen analysis is useful for screening sperm concentration, motility, and morphology. Semen preparation techniques such as swim-up or Percoll gradient centrifugation provide addition information about semen quality [1] and aim at isolating motile sperm for in vitro fertilization (IVF) or for intracytoplasmic sperm injection (ICSI). At present, selection of sperm for ICSI involves the pickup of a motile, morphologically normal-appearing sperm, if possible. Alternatively, we have demonstrated that the hypo-osmotic swelling test can be used to select presumably viable sperm for injection from a semen sample with 0% motility [2]. A concern with the ICSI procedure is the potential risk of injecting an abnormal sperm since the physiologic selection processes, which may influence which sperm fertilizes the oocyte, are bypassed. Although the overall incidence of chromosomal anomalies in children born as a result of IVF does not appear to be increased [3], there has been a suggestion of higher sex chromosomal abnormalities in babies born following ICSI [4]. Male infertility itself is associated with an increase in gross chromosomal abnormalities [5] and occult or recessive genetic disorders such as cystic fibrosis [6, 7]. In addition, although a sperm is mechanically injected into each mature oocyte, the fertilization rate, in general, does not exceed 65% in most clinics [8–10]. Previous studies [11–14] have demonstrated that some sperm in poor-quality semen samples may contain partially decondensed chromatin or DNA strand breakage. The objective of the present study was to evaluate semen samples for sperm DNA fragmentation and to correlate the percentage of abnormal sperm detected with routine semen analysis parameters and with fertilization rates in IVF. We demonstrate that up to 40% of sperm from semen samples obtained in an infertility clinic may contain fragmented DNA.

MATERIALS AND METHODS

Sample Collection

A total of 298 semen samples were collected from couples undergoing IVF or from men examined in the andrology and infertility clinics at The Toronto Hospital, General Division. Written consent for use of the sperm for research was obtained from the patients according to guidelines established by The Toronto Hospital committee for IVF research on human subjects. In addition, we performed a retrospective chart review to obtain information concerning smoking history and days of abstinence prior to production of the sample.

Semen Preparation

Semen samples were collected after at least 48 h of abstinence. After approximately 30 min of liquefaction at room temperature, both routine semen analysis and swim-up assessment were performed using standard techniques [5]. For swim-up, the sample was diluted with Ham's F-10 medium (Gibco, Life Technologies, Grand Island, NY) supplemented with 10% maternal serum or human serum albumin (Cutter Biological, Elkhart, IN). The diluted semen was washed twice by centrifugation for 10 min at 220 × *g*; the final pellet was resuspended in approximately 200 μl of medium and layered gently under 1 ml of fresh medium (supplemented with 10% human serum albumin or maternal serum). The motile spermatozoa were allowed to swim up for 1 h into the overlaying medium at 37°C in a 5% CO_2 incubator. The supernatant (containing swim-up sperm) was aspirated, and the motile sperm concentration was evaluated using a hemocytometer.

Accepted October 14, 1996.

Received May 30, 1996.

[1]This work is supported by a grant from the Medical Research Council of Canada, Ottawa, Ontario, Canada. A.J. is supported by a studentship from Genesis Research Foundation.

[2]Correspondence: Robert F. Casper, 6–246EN, The Toronto Hospital, General Division, 200 Elizabeth St., Toronto, ON, Canada M5G 2C4. FAX: (416) 340–4022; e-mail: r.casper@utoronto.ca

TUNEL Assay

DNA fragmentation in the sperm recovered by swim-up was measured using a modification of the method of terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin end-labeling (TUNEL) described previously [15]. Briefly, 3–4 h after swim-up preparation, about 2×10^6 sperm were fixed with 1% formaldehyde (BDH Inc., Toronto, ON, Canada) for 10 min at room temperature. The sample was centrifuged at $10\,000 \times g$ for 4 min. After washing in PBS (pH 7.4), the sperm were resuspended in 100 μl prewash buffer containing single-strength One-Phor-All buffer (100 mM Tris-acetate, 100 mM magnesium acetate, 500 mM potassium acetate; Pharmacia LKB Biotech, Piscataway, NJ) and 0.1% Triton X-100 (Caledon Laboratories Ltd., Georgetown, ON, Canada) for 10 min at room temperature.

Fixed sperm were spun out of buffer and resuspended in 50 μl of TdT buffer containing 3 μM biotin-16-dUTP (Boehringer Mannheim, Laval, PQ, Canada), 12 μM dATP (Pharmacia LKB Biotech), 0.1% Triton X-100, and 10 U of TdT enzyme (Pharmacia LKB Biotech) and incubated at 37°C for 60 min. After two washes in PBS, the fixed, permeabilized sperm were resuspended in 100 μl of staining buffer consisting of 0.1% Triton X-100 and 1% streptavidin/Texas red anti-biotin (Calbiochem-Novabiochem Corporation, La Jolla, CA) and incubated at 4°C in the dark for 30 min. The stained cells were washed in PBS/0.1% Triton X-100 before analysis on the flow cytometer.

For negative controls, the enzyme terminal transferase was omitted from the reaction mixture. For positive controls, the samples were pretreated with 0.1 IU DNase I (Pharmacia LKB Biotech) for 30 min at room temperature and then labeled.

Flow Cytometry

Red fluorescence was measured at 650 nm using the FL-3 detector of a FACScan flow cytometer (Becton Dickinson, San Jose, CA) equipped with a 15-mW argon-ion laser for excitation. The flow rate during analysis was controlled at approximately 500 cells/sec, and 10 000 sperm were analyzed in each sample. Light-scatter and fluorescence data were obtained at a fixed gain setting in the logarithmic mode. Debris were gated out based on the Forward Scatter vs. Side Scatter dot plot by drawing a region enclosing the cell population of interest, and 10 000 events were collected. The data were initially processed on a computer equipped with LYSIS II software (Becton Dickinson). For further analysis, the fluorescence intensity of each particle analyzed was converted to a DOS text file using HP-Reade software (Verity Software House, Inc., Topsham, ME). The data were analyzed using WinMDI Software (Joseph Trotter, La Jolla, CA). The percentage of labeled sperm in each sample was determined.

TABLE 1. Spearman rank order correlation of semen and demographic variables with percentage of DNA fragmentation determined by flow cytometry.

Variable	n	Coefficient	p value[a]
Conc sperm/mL	222	−0.18	0.008
Motility	298	−0.12	0.04
Fertilization rate	143	−0.16	0.05
Cleavage rate	143	−0.20	0.015
Male age	291	−0.06	NS
Abstinence days	148	−0.003	NS

[a] NS, not significant.

Statistical Evaluation

Sigmastat (Jandel Corporation, San Raphael, CA) was used for data analysis. Since the data were not normally distributed, we used Spearman rank order correlation to determine the correlation of DNA fragmentation with semen analysis or IVF parameters. Smoking vs. nonsmoking data, which also were not normally distributed, were analyzed by the Mann-Whitney U-test. In addition, we used chi-square for analysis of the association between the above parameters with ≤ 4% or > 4% DNA fragmentation. A difference of $p < 0.05$ was considered significant.

RESULTS

A total of 298 male partners provided 298 sperm samples from the IVF program (n = 143) or sperm wash assessment for infertility (n = 155). These samples were processed as described above for TUNEL and flow cytometry. An example of the degree of red fluorescence obtained with the technique is shown in Figure 1. The negative control sperm sample, in which terminal transferase was omitted, demonstrated 0% red fluorescence on FACS (Fig. 2). The positive control sperm sample, which was preincubated with DNase I, showed 82.6% labeled sperm on FACS (Fig. 2). The percentage of sperm with fragmented DNA was less than 4% in the majority of samples but ranged from 5% to 40% in approximately 27% of samples. Using the Spearman rank correlation coefficient, a negative correlation was found between the percentage of sperm with DNA fragmentation and the sperm concentration per milliliter ($r = -0.18$, $p = 0.008$, n = 222; Table 1), motility of the ejac-

TABLE 2. Association between sperm motility, morphology, and concentration and DNA fragmentation of ≤ 4% or > 4% assessed by TUNEL and flow cytometry.

Motility[a]	0–19%	20–39%	40–59%	60–79%	80–100%
≤4% DNA fragment	2	8	35	37	9
>4% DNA fragment	1	10	53	131	12
Morphology[b]	**0–19%**	**20–39%**	**40–59%**	**60–79%**	**80–100%**
≤4% DNA fragment	0	2	18	48	13
>4% DNA fragment	0	3	21	129	51
Concentration[c] (million/ml)	**0–5**	**6–10**	**11–15**	**16–20**	**>20**
≤4% DNA fragment	3	4	5	3	58
>4% DNA fragment	1	2	4	11	131

[a] $\chi^2 = 14.3$, $p < 0.007$.
[b] $\chi^2 = 8.6$, $p < 0.04$.
[c] $\chi^2 = 9.7$, $p < 0.05$.

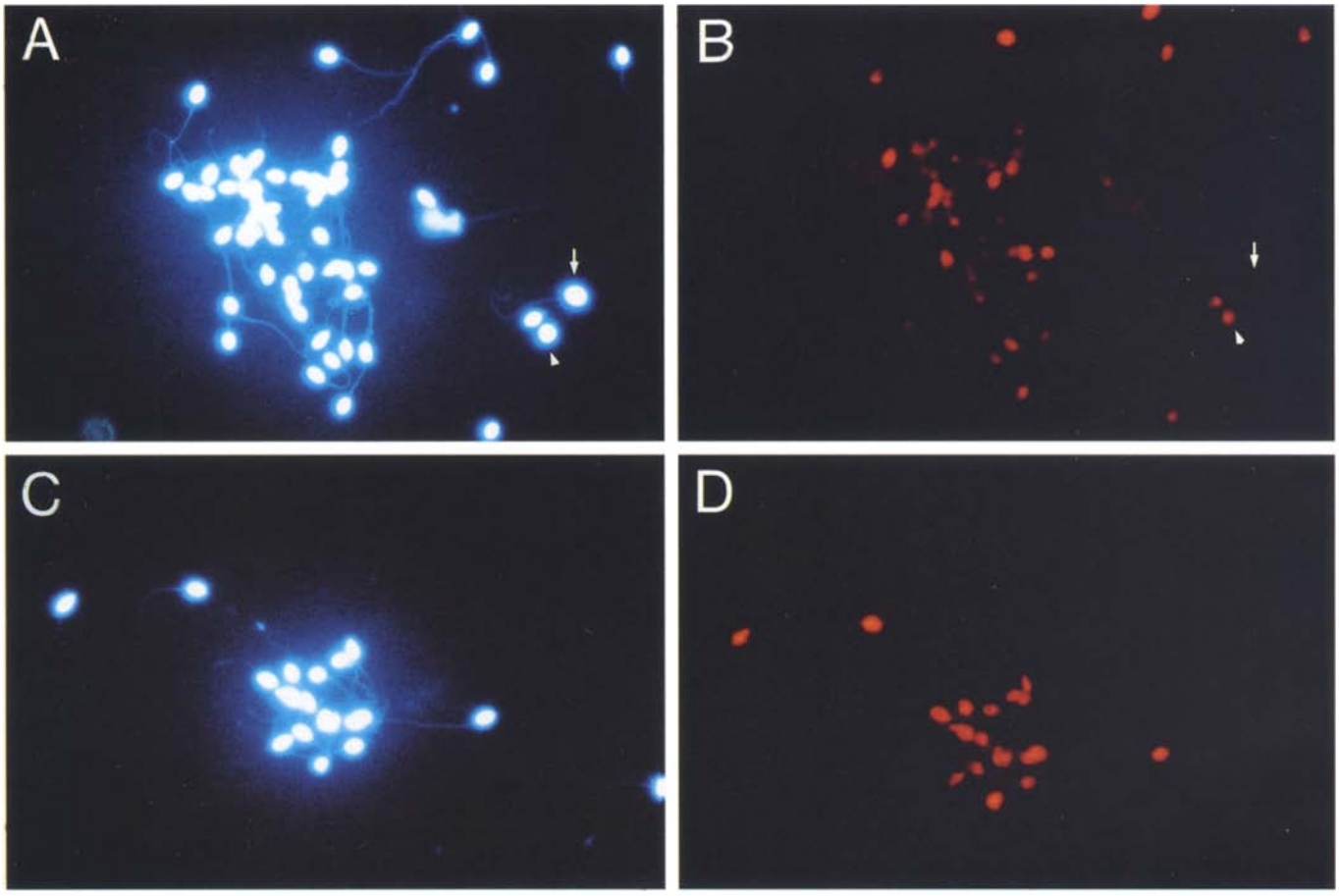

FIG. 1. Human spermatozoa after swim-up analyzed for chromatin status and DNA integrity. **A, B**) Untreated spermatozoa from patient undergoing IVF. Arrows points at unlabeled, normal sperm head with intact DNA. **A**) Sperm were stained with the DNA dye (DAPI). **B**) The same field after TUNEL using biotinylated nucleotides and streptavidin Texas-red conjugate. Arrowhead points at labeled spermatozoa indicating widespread DNA fragmentation. About 40% of sperm were labeled in this sample when subjected to flow cytometry. **C, D**) After fixation, spermatozoa were pretreated with DNase I, which served as a positive control for functionality of TUNEL. **C**) DAPI-stained sperm. **D**) The same field after TUNEL. All sperm are labeled due to activity of DNase. ×1000.

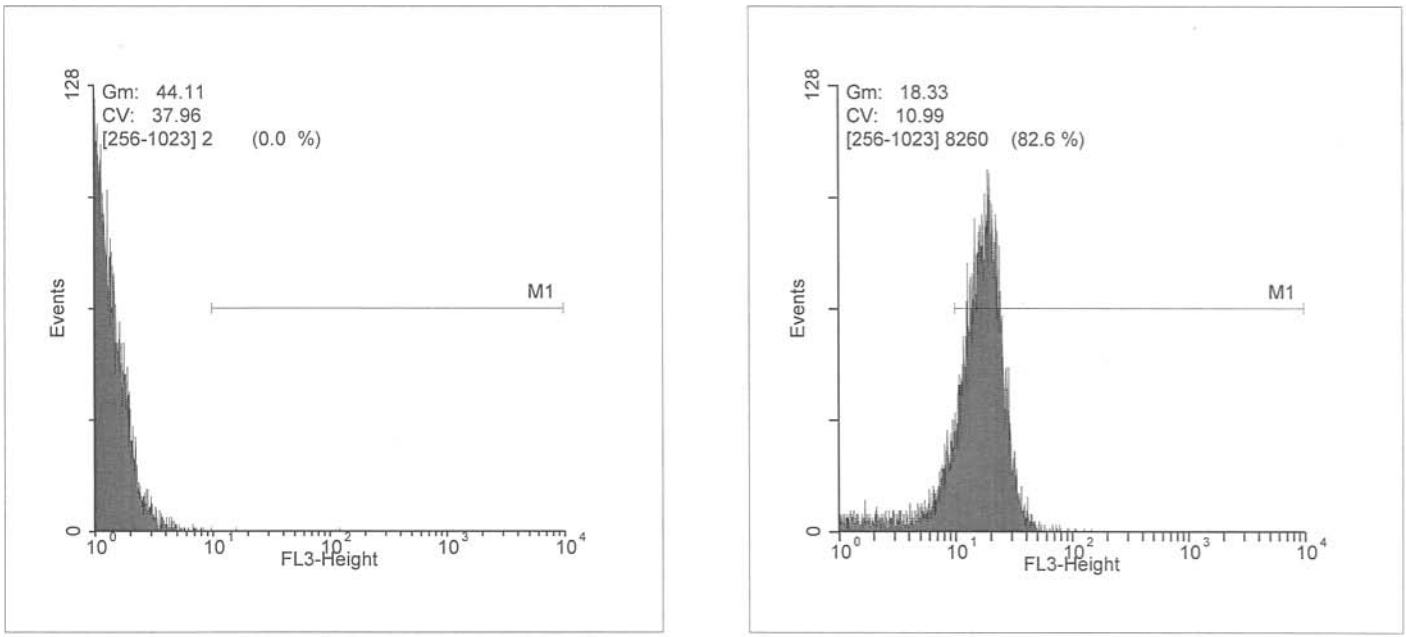

FIG. 2. An illustration of the typical frequency histograms obtained on flow cytometry with markers (M1) for detection of fluorescence at 650 nm. A typical negative control, in which 0% of the sperm are labeled, is shown on the left, and a positive control treated with DNase 1, resulting in 82.6% labeling, is shown on the right.

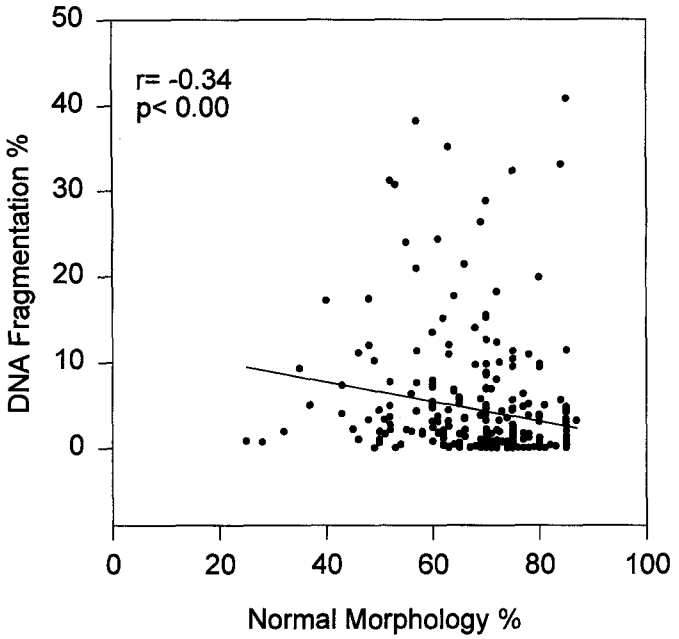

FIG. 3. The correlation between the percentage of sperm with DNA fragmentation on flow cytometry and the percentage normal morphology ($r = -0.34$, $p < 0.00$; n = 285) in the original ejaculated semen samples.

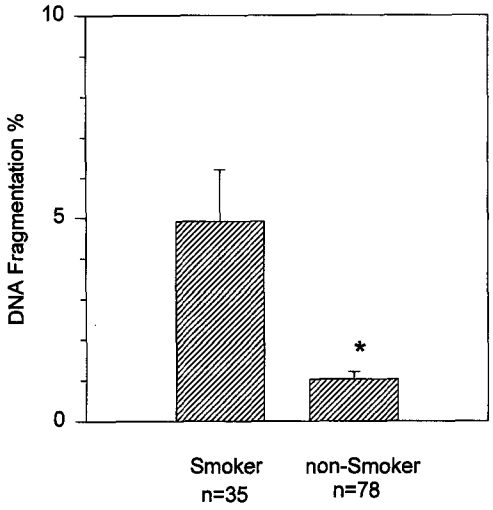

FIG. 4. Mean (± SEM) percentage of sperm with fragmented DNA in 36 men who smoked compared to 78 nonsmokers. * Indicates significance at $p < 0.01$).

ulated sperm ($r = -0.12$; $p = 0.04$; n = 298; Table 1), and percentage normal morphology ($r = -0.34$, $p = 0.00$; n = 285; Fig. 3). In contrast, there was no correlation between percentage of sperm with DNA fragmentation and male age or days of abstinence (Table 1). In 143 IVF samples, a negative correlation was found between the percentage of sperm with DNA fragmentation and the fertilization rate ($r = -0.16$; $p < 0.05$; Table 1) as well as with the embryo cleavage rate ($r = -0.20$; $p < 0.02$; Table 1).

The data described above were further divided into contingency tables illustrating the number of sperm samples with ≤ 4% or > 4% DNA fragmentation for five different categories (0–19%, 20–39%, 40–59%, 60–79%, and 80–100%) for each of the parameters except sperm concentration, for which the categories were 0–5, 6–10, 11–15, 16–20, and > 20 million/ml. As shown in Table 2, significantly more sperm samples had ≤ 4% DNA fragmentation as the percentage of concentration ($\chi^2 = 9.7$, $p < 0.05$), motility ($\chi^2 = 14$, $p < 0.007$), and normal morphology ($\chi^2 = 8.6$, $p < 0.04$) in the samples increased. Similarly, sperm samples with ≤ 4% DNA fragmentation were associated with increased fertilization and embryo cleavage rates ($\chi^2 = 14$, $p = 0.008$ and $\chi^2 = 13$, $p = 0.01$, respectively; Table 3).

No correlation with embryo quality was found (data not shown). Of the male partners in the IVF program, the smoking history was obtained in 113 men, of whom 35 were current smokers and 78 were nonsmokers. We observed a statistically significant relationship between DNA fragmentation and smoking. The 35 men who smoked had an increased percentage of sperm with fragmented DNA (4.7 ± 1.2%) as compared to 78 nonsmokers (1.1 ± 0.2%; $p < 0.01$; Fig. 4).

DISCUSSION

The present study used specific activity of TdT to incorporate biotinylated deoxyuridine to 3′-OH ends of DNA. The signal was amplified by streptavidin-Texas red conjugate, and it is anticipated that signal intensity will increase with an increasing number of DNA breaks. Sperm with normal DNA therefore have only background fluorescence, while those with fragmented DNA (multiple chromatin 3′-OH ends) will fluoresce brightly, enabling identification and sorting by flow cytometry. We have previously used the same technique, along with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) staining of chromatin, to confirm the occurrence of DNA fragmentation associated with apoptosis in fragmented human embryos [15].

Sperm nuclear chromatin is highly condensed compared to that of other cells because of the replacement of histones by protamine and increased disulfide bond formation [16]. As a result, sperm DNA is normally very resistant to physical or chemical denaturation. After citric acid denaturing stress, acridine orange staining revealed fewer sperm with double-stranded DNA in men who had low fertilization in IVF compared to those with apparently normal fertilization [17], suggesting fewer disulfide bonds in the sperm chromosomes. Similarly, increased aniline blue staining of sperm, indicating the persistence of histones, has been reported in infertile men [18], also suggesting increased DNA instability and sensitivity to denaturing stress. Bianchi et al. [13] used fluorochrome staining of sperm from fertile and infertile donors to demonstrate apparent protamine deficiency in some sperm. A similar proportion of sperm in the same samples were positive in DNA nick-translation experiments, suggesting indirectly that protamination may protect against DNA damage. Other recent publications

TABLE 3. Association between fertilization and cleavage rates in IVF and DNA fragmentation of ≤ 4% or > 4% assessed by TUNEL and flow cytometry.

DNA fragmentation	0–19%	20–39%	40-59%	60–79%	80–100%
Fertilization[a]					
≤4%	9	0	8	4	8
>4%	10	13	37	29	25
Cleavage[b]					
≤4%	8	5	4	7	7
>4%	8	8	34	30	32

[a] $\chi^2 = 14$, $p = 0.008$.
[b] $\chi^2 = 13$, $p = 0.01$.

have demonstrated a correlation between the percentage of sperm cells with DNA denaturation after acid exposure and the percentage of sperm with DNA fragmentation [12, 14].

The results of the present study demonstrated that semen samples with poor semen analysis parameters and poor fertilization and cleavage rates in IVF have increased proportions of sperm with DNA fragmentation. This observation may be important in relation to the ICSI procedure, developed to treat severe male factor infertility associated with extremes of abnormal sperm motility, concentration, and/or morphology. Our study suggests that sperm for oocyte injection may often be selected from a sperm population with a relatively high incidence of fragmented DNA. The result of intracytoplasmic injection of a sperm with DNA fragmentation is unknown, but we speculate that some cases of failure of fertilization could be explained on this basis. A recent study by Flaherty and colleagues [19] demonstrated the presence of condensed and decondensed sperm heads in 10% and 71% of unfertilized ICSI oocytes, respectively. Sakkas et al. [20] demonstrated that ICSI performed with sperm samples containing > 30% fluorochrome labeling and > 10% endogenous DNA nicks resulted in unfertilized oocytes in which about half of the sperm remained decondensed. However, oocyte cytoplasmic abnormalities must also be considered in failure of male pronuclear formation.

The reason for sperm DNA fragmentation is also unclear. It is known that reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide can lead to DNA fragmentation in somatic cells [21, 22]. Pathologic processes such as varicocele and infection with pyospermia may increase the level of ROS [23]. Therefore, one could speculate that increased concentrations of ROS, especially in the presence of reduced protamination as previously described [13, 17, 18], may be associated with both poor semen quality and sperm DNA fragmentation. The use of ROS scavengers such as reduced glutathione has been demonstrated to have positive effects on sperm parameters after administration both in vivo [24] and in vitro [25]. An interesting follow-up to the present study would be to measure ROS in semen and to determine whether the concentration correlated with the degree of DNA fragmentation.

Another potential cause of DNA fragmentation is exposure to environmental toxins. The area of environmental pollution by industrial chemicals and declining semen quality has been one of heated debate recently. However, few hard data are available to support the concept of a widespread negative impact of environmental contaminants on semen analysis results. Our finding of a significantly increased percentage of sperm with fragmented DNA in the group of men who smoked as compared to the nonsmokers, however, may suggest a possible link to environmental toxins. In an earlier study, we demonstrated increased follicular fluid concentrations of cadmium in women who smoke compared to nonsmokers [26]. In addition, we demonstrated that women who smoke have a higher incidence of diploid oocytes and triploid zygotes [27], likely as a result of mitotic spindle damage and failure of polar body extrusion. Therefore, smoking appears to have a negative impact on both male and female gametes, and these results support previous findings suggesting decreased fecundity in couples who smoke [28, 29].

In summary, we have demonstrated that a negative correlation exists between the percentage of sperm with DNA fragmentation and routine semen analysis parameters in infertile men. In addition, smoking appears to increase the percentage of sperm with DNA damage. Not surprisingly, we demonstrated that semen samples containing a high percentage of sperm with fragmented DNA were less likely to fertilize in IVF and less likely to result in embryo cleavage than samples with little or no DNA fragmentation. Since men with poor semen analysis parameters or men whose sperm fail to fertilize in IVF are usual candidates for ICSI, it follows that a significant proportion of sperm injected into the oocytes may contain fragmented DNA and may explain the inability of most clinics to achieve a fertilization rate of more than 65% with ICSI. Further studies are required to examine this latter possibility and to develop a method to select sperm with intact DNA for use in ICSI.

REFERENCES

1. Ben-Chetrit A, Senoz S, Greenblatt EM, Casper RF. In vitro fertilization outcome in the presence of severe male factor infertility. Fertil Steril 1995; 63:1032–1037.
2. Casper RF, Meriano J, Jarvi K, Cowan L, Lucato M. The hypo-osmotic swelling test for selection of viable sperm for intracytoplasmic sperm injection in men with complete asthenozoospermia. Fertil Steril 1996; 65:972–976.
3. FIVNAT. Pregnancies and births resulting from in vitro fertilization: French national registry, analysis of data 1986 to 1990. Fertil Steril 1995; 64:746–756.
4. In't Veld P, Brandenburg H, Verhoeff A, Dhont M, Los F. Sex chromosomal abnormalities and intracytoplasmic sperm injection. Lancet 1995; 346:773.
5. Jaffe T, Oates RD. Genetic abnormalities and reproductive failure. Urol Clin North Am 1994; 21:389–408.
6. Patrizio P, Asch RH, Handelin B, Silber SJ. Aetiology of congenital absence of vas deferens: genetic study of three generations. Hum Reprod 1993; 8:215–220.
7. Oates RD, Amos JA. The genetic basis of congenital bilateral absence of the vas deferens and cystic fibrosis. J Androl 1994; 15:1–8.
8. Svalander P, Forsberg AS, Jakobsson AH, Wikland M. Factors of importance for the establishment of a successful program of intracytoplasmic sperm injection treatment for male infertility. Fertil Steril 1995; 63:828–837.
9. Palermo GD, Cohen J, Alikani M, Adler A, Rosenwaks Z. Development and implementation of intracytoplasmic sperm injection (ICSI). Reprod Fertil Dev 1995; 7:211–218.
10. Payne D, Matthews CD. Intracytoplasmic sperm injection—clinical results from the reproductive medicine unit, Adelaide. Reprod Fertil Dev 1995; 7:219–227.
11. Evenson DP, Darzynkiewicz Z, Melamed MR. Relation of mammalian sperm chromatin heterogeneity to fertility. Science 1980; 240:1131–1133.
12. Gorczyca W, Traganos F, Jesionowska H, Darzynkiewicz Z. Presence of DNA strand breaks and increased sensitivity of DNA in situ to denaturation in abnormal human sperm cells: analogy to apoptosis of somatic cells. Exp Cell Res 1993; 207:202–205.
13. Bianchi PG, Manicardi D, Bizzaro D, Bianchi U, Sakkas D. Effect of deoxyribonucleic acid protamination on fluorochrome staining and in situ nick-translation of murine and human spermatozoa. Biol Reprod 1993; 49:1083–1088.
14. Sailer BL, Jost LK, Evenson DP. Mammalian sperm DNA susceptibility to in situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. J Androl 1995; 16:80–87.
15. Jurisicova A, Varmuza S, Casper RF. Programmed cell death and human embryo fragmentation. Mol Hum Reprod 1996; 2:93–98.
16. Balhorn R. A model for the structure of chromatin in mammalian sperm. J Cell Biol 1982; 93:298–305.
17. Tejada RI, Mitchell JC, Norman A, Marik JJ, Friedman S. A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. Fertil Steril 1984; 42:87–91.
18. Foresta C, Zorzi M, Rossato M, Barotto A. Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in infertile men. Int J Androl 1992; 15:330–337.
19. Flaherty SP, Payne D, Swann NJ, Matthews CD. Assessment of fertilization failure and abnormal fertilization after intracytoplasmic sperm injection (ICSI). Reprod Fertil Dev 1995; 7:197–210.
20. Sakkas D, Urner F, Bianchi PG, Bizarro D, Wagner I, Jaquenoud N, Manicardi G, Campana A. Sperm chromatin anomalies can influence

decondensation after intracytoplasmic sperm injection. Hum Reprod 1996; 11:837–843.
21. Buttke TM, Sandstrom PA. Oxidative stress as a mediator of apoptosis. Immunol Today 1994; 15:7–10.
22. Ratan PR, Murphy TH, Baraban JM. Oxidative stress induces apoptosis in embryonic cortical neurons. J Neurochem 1994; 62:376–379.
23. Lenzi A, Picardo M, Gandini L, Lombardo F, Terminali O, Passi S, Dondero F. Glutathione treatment of dyspermia: effect on the lipoperoxidation process. Hum Reprod 1994; 9:2044–2050.
24. Lenzi A, Culasso F, Gandini L, Lombardo F, Dondero F. Placebo controlled, double blind, cross-over trial of glutathione therapy in male infertility. Hum Reprod 1993; 8:1657–1662.
25. Griveau JF, LeLannou D. Effects of antioxidants on human sperm preparation techniques. Int J Androl 1994; 17:225–231.
26. Zenzes MT, Krishnan S, Krishnan B, Zhang H, Casper RF. Cadmium accumulation in follicular fluid of women in in vitro fertilization-embryo transfer is higher in smokers. Fertil Steril 1995; 64:599–603.
27. Zenzes MT, Wang P, Casper RF. Cigarette smoking may affect meiotic maturation of human oocytes. Hum Reprod 1995; 10:3213–3217.
28. Laurent SL, Thompson SJ, Addy C, Garrison CZ, Moore EE. An epidemiological study of smoking and primary infertility in women. Fertil Steril 1992; 57:565–572.
29. Joffe M, Li Z. Male and female factors in infertility. Am J Epidemiol 1994; 140:921–929.