

The Relationship Between Human Semen Characteristics and Sperm Apoptosis: A Pilot Study

ZUYING CHEN,* RUSS HAUSER,*† ALEXANDER M. TRBOVICH,* JAN L. SHIFREN,* DAVID J. DORER,* LINDA GODFREY-BAILEY,† AND NARENDRA P. SINGH‡

From the *Vincent Memorial Obstetrics & Gynecology Service, Andrology Laboratory and In Vitro Fertilization Unit, Massachusetts General Hospital, Boston, Massachusetts; the †Department of Environmental Health, Occupational Health Program, Harvard School of Public Health, Boston, Massachusetts; and the ‡Department of Bioengineering, University of Washington, Seattle, Washington.

ABSTRACT: This work was undertaken to explore the association between human semen characteristics and apoptosis in ejaculated sperm. We collected semen samples from 23 consecutive male patients who presented to the Andrology Laboratory at Massachusetts General Hospital (MGH) for routine semen analysis. Sperm concentration and motility were measured using computer-assisted sperm analysis. Morphology was assessed using Tygerberg strict criteria. The DNA diffusion assay was used to assess the percentage of apoptosis in ejaculated sperm. In this assay, cells were mixed with agarose and placed into a microgel on a microscopic slide. The cells were stained with YOYO-1 dye, and apoptotic cells were viewed under a fluorescent microscope. Among 23 men, the mean (SD) sperm concentration, percent motility, percent progressive motility,

and normal morphology were 125.5 (92.3) million/mL, 45.6% (22.2), 28.4% (15.2), and 8.0 (4.6), respectively. The mean (SD) percent of apoptosis in ejaculated sperm was 8.3% (6.2) with a range from 1.1% to 20.1%. There were inverse associations between percent apoptosis and sperm motility ($P = .0025$), progressive motility ($P = .0051$), and morphology with a normal or good pattern of fertilization by Kruger strict criteria ($P = .0045$), and a positive relationship between percent apoptosis and sperm tail defects ($P = .0053$). In ejaculated semen, the percent sperm apoptosis was associated with several measures of semen quality.

Key words: Semen quality, sperm motility, sperm morphology, DNA diffusion assay.

J Androl 2006;27:112–120

Conventional semen analyses provide information on the clinical status of male fertility (Keel and Webster, 1990; WHO, 1999). Semen analysis includes assessment of volume, color, viscosity, pH, concentration, motility, and morphology (WHO, 1999). However, the results of semen analysis are only moderately predictive of an individual's fertility (Jeyendran, 2000). Since male factor infertility is solely responsible in about 20% of infertile couples and contributory in another 30%–40% (Thonneau et al, 1991), it is important to develop new methodology and assays to improve the clinical diagnosis of male infertility and to provide knowledge about sperm function.

Sperm DNA damage and sperm apoptosis have been considered as potentially useful indices of male fertility. Cellular apoptosis is a normal event that occurs both during and after embryonic development. Germ cell loss

(Roosen-Runge, 1973; Allan et al, 1987), now recognized as occurring via apoptosis, is a dominant process during spermatogenesis and is regulated by p53, p21, caspases, bcl-2, and Fas expression levels, with many alternate pathways (Lee et al, 1997; Sinha Hikim and Swerdloff, 1999; Print and Loveland, 2000; Martincic et al, 2001; Anzar et al, 2002; Said et al, 2004). It has been found that the number of sperm with Fas expression was low in subjects with normal sperm parameters but high in men with abnormal sperm parameters (Sakkas et al, 1999)

Mild to moderate genotoxic and cytotoxic insults to germ cells also induce apoptosis. Unlike somatic cells, it is unclear whether ejaculated sperm retain the capacity to undergo apoptosis (Sakkas et al, 1999; Wang et al, 2003; Lachaud et al, 2004). Male infertility appears to be positively correlated with increased levels of sperm with apoptotic markers (Oehninger et al, 2003; Paasch et al, 2003; Wang et al, 2003). The presence of immature sperm may contribute to increased levels of apoptotic markers in ejaculated sperm (Cayli et al, 2004). Although apoptosis is considered a mechanism to ensure selection of sperm cells with undamaged DNA, sperm with DNA damage that are not eliminated by apoptosis may fertilize an ovum (Sun et al, 1997; Morris et al, 2002).

Necrosis and apoptosis are 2 forms of cell death. In

Supported by grants ES09718 and ES00002 from the National Institute of Environmental Health Sciences.

Correspondence to: Dr Russ Hauser, Department of Environmental Health, Occupational Health Program, Harvard School of Public Health, Building 1, Room 1405, 665 Huntington Ave, Boston, MA 02115 (e-mail: rhauser@hohp.harvard.edu).

Received for publication April 19, 2005; accepted for publication September 16, 2005.

DOI: 10.2164/jandrol.05073

apoptotic cells, the plasma membranes are intact, whereas in necrotic cells, the plasma membranes lose their integrity and become leaky (Vermees et al, 1995). Apoptosis is physiologically programmed cell death that affects single cells without any associated inflammation in the surrounding tissues (Wyllie et al, 1980). Apoptosis is characterized by a distinct series of ultrastructural morphological (Wyllie et al, 1980) and biochemical changes (Wyllie et al, 1980; Williams and Smith, 1993). There is chromatin aggregation, cytoplasmic condensation, and indentation of nuclear and cytoplasmic membranes in apoptotic cells (Aznar et al, 2002). At the end of this process, the nucleus undergoes fragmentation, and the whole cell blebs and fragments into apoptotic bodies (Aznar et al, 2002).

While there have been many published studies investigating the relationship between traditional semen parameters and apoptosis, the results vary and firm conclusions are still outstanding (Sun et al, 1997; Gandini et al, 2000; Oosterhuis et al, 2000; Shen et al, 2002). Two commonly used methods for detecting spontaneous and induced apoptosis in sperm are the TUNEL assay (Baccetti et al, 1996; Weil et al, 1998; Gandini et al, 2000; Muratori et al, 2000; Oosterhuis et al, 2000; Hoyes et al, 2001; Koji, 2001; Ricci et al, 2002; Sakkas et al, 2002; Shen et al, 2002; Weng et al, 2002) and annexin V/PI staining (Hoyes et al, 2001; Ricci et al, 2002; Shen et al, 2002; Weng et al, 2002).

In the present study, we explored the associations between semen characteristics and the percent of apoptosis in ejaculated sperm in male partners of infertile couples. The DNA diffusion assay was used to assess sperm apoptosis, as it has been shown to be a simple and reproducible assay (Singh, 2000). This assay is based on the principle that apoptotic cells have numerous alkali-labile sites (Singh, 2000), and these sites, once exposed to alkaline conditions, yield low-molecular-weight DNA fragments. These pieces can easily diffuse in the agarose matrix giving the appearance of a hazy halo around the cell. This unique pattern of DNA gradient diffused in agarose is characteristic of apoptotic cells and is distinguishable from necrotic cells (Singh, 2000).

Materials and Methods

Participants

The Human Subject Committee Internal Review Board of the Massachusetts General Hospital (MGH) and Harvard School of Public Health approved the study protocol. Study subjects were male partners of couples who presented to the Vincent Memorial Andrology Laboratory at MGH between April 13, 2003, and April 18, 2003, for an evaluation of infertility. Discarded semen

was used for the apoptosis assay. Age of participants ranged from 28 to 47 years old.

Collection of Semen Samples

Semen was collected by masturbation into a sterile plastic specimen cup at the hospital. Subjects were instructed to abstain from ejaculation for at least 48 hours and no longer than 7 days before producing the semen sample. The sample was liquefied for at least 20 minutes, but no longer than 1 hour, before performing a routine semen analysis that included volume, pH, sperm concentration, sperm motility, progressive motility, and sperm morphology. A portion of the discarded semen was then used for the DNA diffusion assay. The assay was performed on the discarded semen within 1.5 hours after receiving the specimen.

Laboratory Evaluation

Concentration and Motility—Semen samples were analyzed for sperm concentration and motion parameters by computer-aided semen analyzer (CASA, HTM-IVOS Version 10; HTM-IVOS, Beverly, Mass). Setting parameters and the definition of measured sperm motion parameters for CASA were established by Hamilton Thorn Company. To measure both sperm concentration and motility, 5 μ L of semen from each sample was placed into a prewarmed (37°C) Makler counting chamber (Sefi-Medical Instruments, Haifa, Israel). A minimum of 200 sperm cells from at least 4 different fields was analyzed from each specimen. Motile sperm was defined as World Health Organization (WHO) grade “a” sperm (rapidly progressive with a velocity greater than or equal to 25 μ m/s at 37°C) plus “b” grade sperm (slow/sluggish progressive with a velocity greater than or equal to 5 μ m/s but less than 25 μ m/s) (WHO, 1999). The progressive motile sperm was defined grade “a” sperm (WHO, 1999).

Morphology—At least 2 slides were made for each fresh semen sample. The resulting thin smear was allowed to air dry for an hour before staining with a Diff-Quik staining kit (Dade Behring AG, Dudingon, Switzerland). Morphological assessment was performed with a Nikon microscope using 10 \times ocular lens combined with a 100 \times oil immersion objective lens (Nikon Company, Tokyo, Japan). A minimum of 200 sperm cells were counted from 2 slides for each specimen. Morphology was assessed using the strict criteria by Kruger et al (1988). The cutoff for morphology with normal or good pattern of fertilization is set at greater than or equal to 4%. Results were expressed as the percentage of normal spermatozoa and abnormal spermatozoa (head defects, midpiece defects, and tail defects). Semen analysis was performed before the DNA diffusion assay.

Endtz Test—The Endtz test is a rapid staining method for differentiation of polymorphonuclear leukocytes from germinal cells (Endtz, 1974). By adding a peroxidase solution to semen, a histochemical reaction occurs, staining the polymorphonuclear leukocytes a brown color. After staining, the specimen is read under microscopy in a Makler chamber and quantified by counting the number of polymorphonuclear leukocytes. Normal concentration of polymorphonuclear leukocytes in semen is less than 1×10^6 /mL (WHO, 1999). A value greater than or equal to 1 million/mL is considered a positive test for polymorphonuclear leukocytes.

DNA Diffusion Assay—A small aliquot of the semen sample

was analyzed for apoptosis using the DNA diffusion assay (Singh, 2000). Briefly, 60 mg of high resolution agarose 3:1 (Amresco, Solon, Ohio) was boiled in 9 mL of distilled water in a microwave oven, and 1 mL of 10× modified phosphate-buffered saline (PBS) was added to it (for one L: 80 g of NaCl, 2 g of KCl, 2 g of KH_2PO_4 , 11.5 g of anhydrous Na_2HPO_4 or 29 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 32 g tris hydrochloride, pH 7.4). After adjusting volume to 10 mL by adding distilled water, the solution was boiled once more. The final concentration of agarose was 0.6%. The agarose was then dispensed in small aliquots and maintained at 55°C for 24 hours before use.

Preparing slides. Microgel electrophoresis (MGE) slides (Eric Scientific Co, Portsmouth, NH) were coated with 50 μL of 0.6% agarose 3:1. The first layer of the microgel was made by pipetting 50 μL of agarose on top of the frosted part of a slide while holding the slide horizontally in the left hand between thumb and index finger and smearing the agarose in one motion using a pipette tip held horizontally by the right hand. This layer was then air dried. The second layer of the microgel was made by mixing approximately 10 000 sperm cells in 5 μL of PBS with 50 μL of 0.6% agarose 3:1 for each slide. Fifty microliters of this mixture was layered onto the slides precoated with first layer of microgel, and this agarose cell mixture was immediately covered with a 24 × 50 mm² #1 cover glass (Corning Glass Works, Corning, NY). The slides were put into a cold steel tray kept on ice. After removing the cover glass, 200 μL of 2% superfine resolution (SFR) agarose solution was layered as before to make a third layer of microgel. Use of 2% SFR agarose is essential for controlling the extent of DNA diffusion from apoptotic cells in agarose. The slide was covered with a cover slip after the third layer was made. After keeping the slides on ice for 2 minutes, cover glasses were removed and the slides were immersed and maintained for 10 minutes in a freshly made cold lysing solution (1.25 M NaCl, 1 mM tetrasodium EDTA, 5 mM Tris, 0.01% sodium lauryl sarcosine, 0.2% dimethylsulfoxide (DMSO) and 300 mM NaOH).

Neutralization, DNA precipitation, and staining of microgels. Slides for neutralization and DNA precipitation were immersed twice in freshly prepared 20 mM Tris, pH 7.4 in 50% ethanol with 1 mg/mL of spermine for 15 minutes. Slides were air dried. One slide at a time was stained with 50 μL 0.25 μM of Yellow-Orange Yellow-Orange-1 (YOYO-1) in 2.5% DMSO and 0.5% sucrose and immediately examined under a fluorescent microscope. Apoptotic cells were scored using a DMLB epifluorescence microscope (Leica, Wetzlar, Germany) having an excitation filter of 490 nm, a 500-nm dichroic filter, and an emission filter of 515 nm, emitting green fluorescence. One thousand cells were scored from each sample, and apoptotic cells were counted using 400× magnification. Sperm exhibiting diffuse halos in the DNA diffusion assay were considered apoptotic. The percentage of apoptotic cells with diffuse DNA and a hazy outline was calculated from a total of 1000 cells counted. The investigator who performed all apoptosis assessments was blinded to the results of the semen analyses.

Statistical Analyses

In univariate regression analyses, log (base 10) percent apoptosis was regressed on age or semen characteristics

in separate regression models. The regression coefficients represent the change on the log scale of apoptosis percent per one unit change in the predictor (ie, age or semen characteristics). For ease of interpretation, regression coefficients were back transformed to represent the multiplicative change in apoptosis percent per one unit change in predictor. Statistical computations were made using the R statistical package (Dalgaard, 2002). The study ($n = 23$ subjects) was powered to detect a correlation of 0.51 at an alpha of .05 with 80% power. One subject was excluded from the primary data analysis because motility and progressive motility of semen were zero percent. However, in analyses including this subject, results were essentially unchanged (data not shown).

Results

The individual semen analysis results for each subject are presented in Table 1. The distributions of the subjects' ages, semen characteristics, and percent apoptosis are shown in Table 2. Subjects' ages ranged from 28 to 47 years, with a mean age of 37.8. The majority of the subjects (57%) were between 30 and 40 years old; only 8% were less than 30 years and 35% were greater than 40 years old. The mean (SD) semen volume was 2.8 mL (1.4). The mean (SD) sperm concentration was 125.5 million/mL (92.3). The mean (SD) percent motility and progressive motility were 45.6% (22.2) and 28.4% (15.2), respectively. The mean (SD) percent normal morphology was 8.0% (4.6). The mean (SD) of Endtz test results was 0.1 (0.2). Only 1 subject had leukocyte counts greater than 1 million/mL (15.2 million/mL). This patient had a 3.4% apoptosis by the DNA diffusion assay. This subject's Endtz test result was excluded from the statistical analysis because it was an outlier (volume 15.2 million/mL). The mean (SD) percent of apoptosis in ejaculated sperm was 8.3% (6.2), with a range from 1.1% to 20.1%. The semen samples had few somatic cells. Some of these somatic cells were also shown to have undergone apoptosis. Somatic cells have twice the amount of DNA as compared to sperm cells; thus, those that had undergone apoptosis had a larger DNA diffusion area. These cells are easily differentiated from sperm cells under the fluorescent microscope. Somatic cells were not scored and are not included in the data analysis.

The results of the regression analyses exploring the relationship between percent apoptosis in ejaculated sperm and patient age and semen characteristics are presented in Table 3. In univariate analyses, 3 semen parameters were inversely associated with percent sperm apoptosis. After back transformation of the regression coefficients, there was -2.38% multiplicative change in apoptosis percent per 1 unit change in percent motility, ($P = .0025$),

Table 1. Individual semen analysis results for each subject ($n = 23$)

Subject	Age, y	Volume, mL	Concentration, $\times 10^6$	Motility, %	Progressive Motility, %	Morphology Normal, %	Apoptosis, %
1	28	5.2	99.6	48	30	7	2.8
2	44	4.4	46.2	11	7	7	8.8
3	34	1.7	307.1	68	38	11	6.7
4	32	3.2	82.2	56	36	11	15.4
5	38	2.5	82.5	21	12	1	17.8
6	39	2.6	130	14	7	3	15
7	35	6.4	224.5	42	24	5	13.3
8	32	2.0	53.8	24	13	5	19.2
9	33	1.1	316.3	66	32	11	2.5
10	45	1.7	92.8	44	33	9	13.6
11	38	4.6	27.7	37	25	5	20.1
12	29	2.1	38.7	14	10	9	11.1
13	44	0.9	14.7	15	15	1	7.5
14	42	2.2	99.3	34	21	8	2.7
15	40	3.1	210.9	66	28	3	3.9
16	45	3.1	154.5	84	59	11	1.9
17	44	2.5	39.1	62	44	14	1.4
18	30	3.6	68.2	68	38	14	1.1
19	34	3.4	60.3	54	41	10	3.4
20	42	3.0	107.5	31	17	7	3.2
21	37	3.0	110.3	80	55	18	3.5
22	47	2.1	285.7	62	50	13	5.7
23	37	0.8	235.3	48	18	1	10.8

a -3.26% multiplicative change in apoptosis percent per 1 unit change in percent progressive motility ($P = .0051$) and a -10.44% multiplicative change in apoptosis percent per 1 unit change in percent normal morphology ($P = .0045$). Figures 1 through 3 show the relationships between these 3 parameters and sperm apoptosis. Sperm tail defects were positively associated with percent sperm apoptosis, there was an 8.83% multiplicative change in apoptosis percent per 1 unit change in percent tail defects ($P = .0053$). Typical halos are demonstrated in apoptotic human sperm cells (Figure 4).

There were no statistically significant associations between percent sperm apoptosis and semen volume ($P = .97$), viscosity ($P = .94$), pH ($P = .90$), sperm concentration ($P = .65$), motile sperm concentration ($P = .21$), progressive motile sperm concentration ($P = .20$), total progressive motile sperm count per sample ($P = .25$), and percent abnormal morphology, head ($P = .57$), and midpiece ($P = .09$). There also was no statistically significant relationship between apoptosis and Endtz test results ($P = .53$). There was also no association between subject age and percent apoptosis ($P = .78$).

Table 2. Distribution of age and semen parameters for the study population ($n = 23$)

Parameter	Mean	SD	Minimum	Maximum
Age (y)	37.8	5.7	28	47
Volume (mL)	2.8	1.4	0.8	6.4
pH	8.5	0.2	7.9	9.0
Concentration (million/mL)	125.5	92.3	14.7	316.3
Motile sperm (million/mL)	66.8	65.7	3.4	209.2
Motility (%)	45.6	22.2	11.0	84.0
Progressive motile sperm (10^6 /mL)	39.5	39.2	3.4	141.9
Progressive motility (%)	28.4	15.2	7.0	59.0
Total progressive motile sperm (10^6)	103.7	101.2	2.0	351.3
Normal morphology (%)*	8.0	4.6	1.0	18.0
Head defects (%)*	65.5	5.7	60	80
Midpiece defects (%)*	12.7	4.0	6	20
Tail defects (%)*	13.9	5.9	6	26
Endtz test (10^6 /mL)	0.1	0.2	0	0.4
Apoptosis (%)	8.3	6.2	1.1	20.1
Apoptosis (\log_{10})	0.8	0.4	0.04	1.3

* Morphological assessment by Tygerberg Kruger strict criteria.

Table 3. Multiplicative factors and P values from univariate regression of apoptosis percent regressed on age or semen characteristics of ejaculated sperm (n = 23)*

Characteristic	Percentage Increase (Decrease) per Unit Change	P
Age (y)	(0.95)	.78
Volume (mL)	(0.61)	.97
Viscosity (1–5)	1.19	.94
pH	(9.74)	.90
Concentration (10 ⁶ /mL)	(0.10)	.65
Motile sperm concentration (10 ⁶ /mL)	(0.36)	.21
Motility (%)	(2.38)	.0025†
Progressive motile sperm concentration (10 ⁶ /mL)	(0.63)	.20
Progressive motility (%)	(3.26)	.0051†
Total progressive motile sperm (10 ⁶)	(0.22)	.25
Morphology normal (%)	(10.44)	.0045†
Head defects (%)	1.97	.57
Mid-piece defects (%)	(7.79)	.09
Tail defects (%)	8.83	.0053†
Endtz test (10 ⁶ /mL)	100.81	.53

* Multiplicative factor represents the change in apoptosis percent per one unit change in age (y) or semen characteristic. For instance, for sperm motility, the multiplicative factor represents a 2.38% multiplicative change in apoptosis percent per unit change in sperm motility.

† $P < .05$.

Nine men (39%) had above reference WHO value semen parameters (sperm concentration greater than 20 million/mL, motility greater than 50%, and progressive motility greater than 25%) and morphology with normal or good pattern of fertilization by Kruger strict criteria (percent normal morphology greater than 4%). Fourteen men (61%) had 1, 2, 3, or all 4 parameters below reference

values. For example, some men had concentrations less than 20 million/mL, while others had motility or progressive motility less than 50% or 25%, respectively, or morphology less than 4%. The percent sperm apoptosis was lower (4.6%) in the group of men (n = 9) with all 4 semen parameters above reference values than it was in the group of men (n = 14) with 1 or more semen parameters below reference values (10.7%).

Discussion

Apoptosis is a mechanism regulating spermatogenesis in humans, and there are clear differences in molecular markers of apoptosis between males with normal sperm parameters and those with abnormal sperm parameters (Sakkas et al, 1999). This is consistent with the results from our study that percent apoptosis was lower (4.6%) in men with semen parameters above reference value compared to men with 1 or more abnormalities (10.7%). Sakkas et al (1999) proposed 2 theories to describe the origin of DNA damage in mature spermatozoa. The first arises from studies performed in animal models and is linked to the manner in which mammalian sperm chromatin is packaged, while the second attributes the nuclear DNA damage in mature spermatozoa to apoptosis. Those individuals with high rates of apoptosis may have an increased percentage of sperm with genetic damage and may have a higher number of sperm that are immotile. It is possible that genetic damage in sperm, apoptosis, and motility are related to each other.

The DNA diffusion assay is a highly sensitive method for apoptosis assessment. Singh observed the sensitivity of the assay to be greater than 98% (Singh, 2000). The

Relationship between percent sperm apoptosis and percent motility

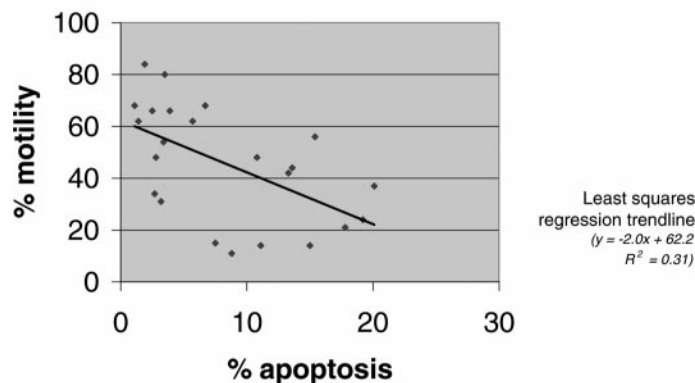


Figure 1. Relationship between percent apoptosis and sperm motility. Note: Least squares regression trend line. Percent of motile sperm was inversely associated with percent sperm apoptosis ($P = .01$).

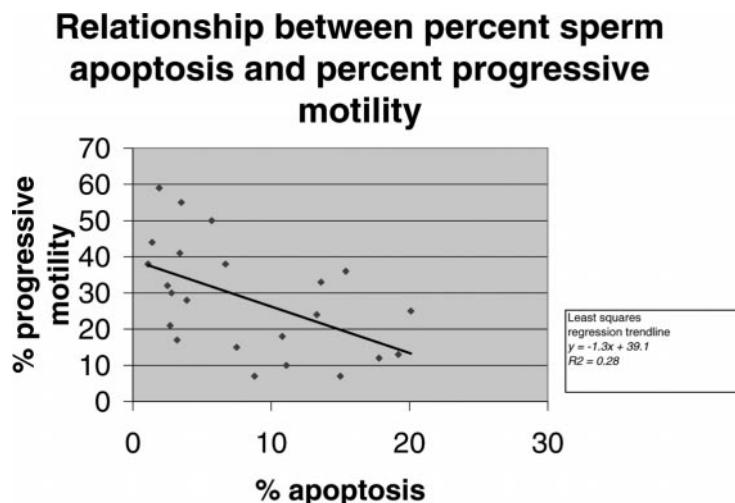


Figure 2. Relationship between percent apoptosis and progressive sperm motility. Note: Least squares regression trend line. Percent of progressive motile sperm was inversely associated with percent sperm apoptosis ($P = .02$).

DNA diffusion assay has previously been used to assess sperm apoptosis (Singh et al, 2003), and results were well within the range observed by other commonly used assays such as TUNEL and annexin V. TUNEL assay, although sensitive, is associated with a number of artifacts, as it labels DNA strand breaks from any insult, in both apoptotic and nonapoptotic cells (Kocks et al 1998). Annexin V assay, based on detection of externalization of phosphatidylserine, may detect normal sperm after capacitation as apoptotic, thus overestimating total apoptotic cells (Kotwicka et al 2002).

The DNA diffusion assay is simple and specific for the detection of apoptosis and necrosis. Apoptotic cell nuclei display hazy halos while necrotic cell nuclei are larger and less defined, with halos that have clear outer borders (Singh, 2000).

In the present study, the mean (SD) percent apoptosis

in ejaculated sperm was 8.3% (6.2), with a range from 1.1 to 20.1. The results are similar to those of Singh et al (2003), who showed 0.3% to 23% apoptosis of human sperm cells (mean, 6.5%) using the DNA diffusion assay. Similarly, using the TUNEL assay, Baccetti et al (1996) reported that 0.1% to 10% of sperm are apoptotic in human ejaculated sperm. Oosterhuis et al (2000) found that 20% of sperm from infertile men were TUNEL positive. Baccetti et al (1996) also reported a variance in the percentage of apoptotic sperm cells of approximately 0.1% in fertile controls, 10% in men with varicocele or infections (including AIDS), 20% in men with cryptorchidism, 25% in immature patients, and up to 50% in testicular seminoma carriers.

The present study found inverse associations between percent apoptosis in ejaculated human semen and sperm motility, progressive motility, and morphology. Similar

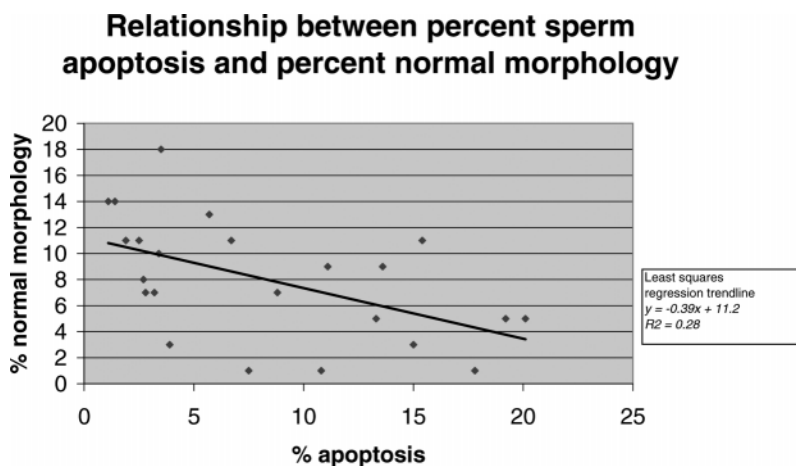


Figure 3. Relationship between percent apoptosis and normal morphology. Note: Least squares regression trend line. Percent of sperm with normal morphology was inversely associated with percent sperm apoptosis ($P = .01$).

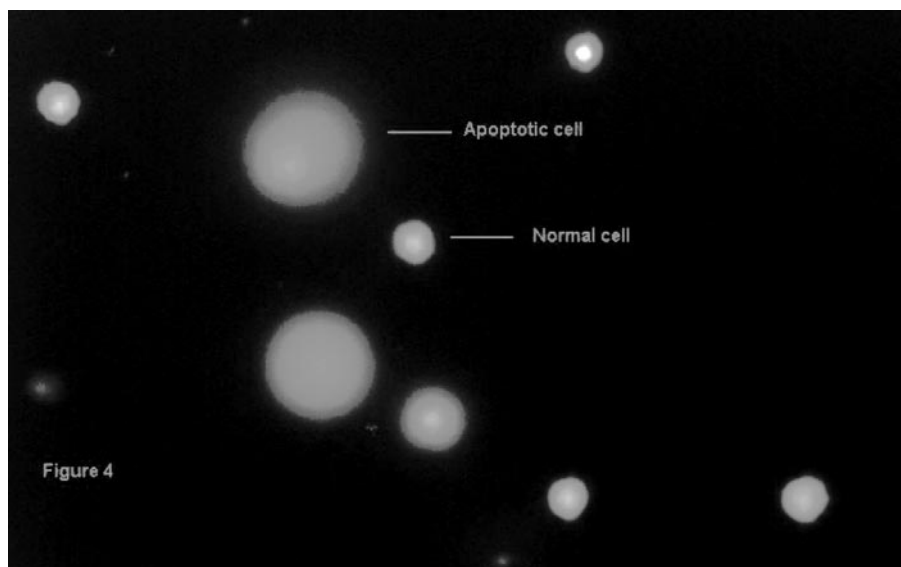


Figure 4. Photograph of apoptotic sperm and normal sperm. Apoptosis assessed by the DNA diffusion assay; 400 \times .

findings were reported by several researchers (Sun et al, 1997; Barroso et al, 2000; Oosterhuis et al, 2000; Shen et al, 2002). In a pilot study, Oosterhuis et al (2000) found a significant inverse correlation between sperm motility and apoptosis by annexin V assay ($r = -.289$; $P < .05$) in ejaculated sperm. A cross-sectional observational study of 10 men from a fertility clinic conducted by Barroso et al (2000) using the gradient separation method reported that the semen fractions with low sperm motility had a significantly higher proportion of cells with DNA damage ($P < .005$) measured by TUNEL assay and monoclonal antibody labeling of single-stranded DNA. A report on 60 men from a fertility clinic in Singapore found that apoptotic cells (Annexin V-positive) were inversely correlated with sperm motility (Shen et al, 2002).

Some studies have been performed to link sperm morphology with the percentage of apoptotic sperm. Two studies found apoptosis positively associated with various forms of abnormal sperm morphology, including defects of the sperm head, midpiece, and tail (Gandini et al, 2000; Shen et al, 2002). Our data showed an inverse correlation between sperm apoptosis and normal or good pattern of fertilization morphology and a positive relationship between sperm tail defects and apoptosis.

In the present study, the percent of apoptosis was not significantly correlated with either sperm concentration or total sperm count. The literature to date has inconsistent results on the relationship between sperm apoptosis and measures of sperm quantity (Sun et al, 1997; Gandini et al, 2000; Oosterhuis et al, 2000; Shen et al, 2002). Oosterhuis et al (2000) found a strongly significant inverse correlation between the percentage of TUNEL-positive cells and the concentration of spermatozoa ($r = -.629$;

$P < .0001$). They hypothesized that a low sperm concentration might be caused by a high rate of apoptosis. In a Canadian study of 298 men attending an infertility program, Sun et al (1997), using the TUNEL assay to assess DNA fragmentation, found a negative correlation with sperm concentration. In contrast, Shen et al (2002) in a study of 60 subfertile men reported that the percentage of apoptosis was significantly positively correlated with both sperm concentration and total sperm count when using the Annexin V and TUNEL assays. It is possible that these inconsistencies will be clarified as further studies are conducted.

Recently, using the DNA diffusion assay, Singh et al (2003) found an age-related decrease in the percent of sperm apoptosis among men of reproductive age. Sixty-six men were recruited; 40 were patients seeking an infertility evaluation, and 26 were controls. These subjects ranged in age from 20 to 57 years. Sperm exhibiting diffuse halos in the DNA diffusion assay were considered apoptotic. Overall, $6.5\% \pm 5.1\%$ of sperm exhibited this pattern (range, 0.3% to 23%). The percent of apoptotic sperm was significantly inversely related to age ($P = .029$). The 2 older age groups (greater than 35 years and greater than 43 years) showed a highly significant decrease in apoptosis compared with the younger group (less than 25 years). However, there was no significant difference when subjects aged less than 25 years were compared with those aged greater than 25 years. In the present study, we did not find a relationship between age and apoptosis. This may be due to small sample size and narrow age range of our study population.

Percentages of apoptosis in human ejaculated sperm may correlate with fertilization rates after in vitro fertil-

ization (IVF) or intracytoplasmic sperm injection (ICSI) procedures (Sun et al, 1997; Levy et al, 2001; Wang et al, 2002). Detection of apoptosis in human sperm could provide additional information and may explain more about the causes of fertilization failures (Levy et al, 2001). In 143 IVF samples, a significant negative association was found between the percentage of sperm with DNA fragmentation, fertilization rate ($P = .008$), and embryo cleavage rate ($P = .01$) (Sun et al, 1997). Wang et al (2002) evaluated the relationship between sperm apoptosis and male infertility. They reported that sperm apoptosis by flow cytometry was significantly different between fertile and infertile groups, with results of 4.28 (± 1.66)% and 18.67 (± 8.55)%, respectively ($P < .01$).

We conclude that the use of the DNA diffusion assay to measure apoptosis in human ejaculated sperm in a clinical andrology laboratory is a simple and potentially useful assessment as part of an infertility evaluation. Our data showed significant relationships between apoptosis and sperm motility, progressive motility, morphology, and tail defects. Based on the findings of our study, additional study is warranted to confirm this association and to evaluate sperm apoptosis in relation to fertilization and pregnancy rates. We anticipate that the DNA diffusion assay for apoptosis in sperm may eventually be used as a diagnostic test, in conjunction with semen analysis, for male factor infertility.

Acknowledgments

The authors thank Ana Trisini (Research Assistant at Harvard School of Public Health, Department of Environmental Health) for assistance with manuscript editing and Nelta Mercedat Lozius (Clinical Laboratory Assistant at Massachusetts General Hospital, Obstetrics & Gynecology Services) for assistance with analysis of the samples.

References

- Allan DJ, Harmon BV, Kerr JFR. Cell death in spermatogenesis. In: Porter CS, ed. *Perspective on Mammalian Cell Death*. Oxford, United Kingdom: Oxford University Press; 1987:229–258.
- Anzar M, He L, Buhr MM, Kroetsch TG, Pauls KP. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biol Reprod*. 2002;66:354–360.
- Baccetti B, Collodel G, Piomboni P. Apoptosis in human ejaculated sperm cells (notulae seminologicae 9). *J Submicrosc Cytol Pathol*. 1996;28:587–596.
- Barroso G, Morshedi M, Oehninger S. Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum Reprod*. 2000;15:1338–1344.
- Cayli S, Sakkas D, Vigue L, Demir R, Huszar G. Cellular maturity and apoptosis in human sperm: creatine kinase, caspase-3 and Bcl-XL levels in mature and diminished maturity sperm. *Mol Hum Reprod*. 2004;10:365–372.
- Dalgaard P. *Introductory Statistics with R*. New York, NY: Springer; 2002.
- Endtz AW. A rapid staining method for differentiation granulocytes from “germinal cells” in Papanicolaou-stained semen. *Acta Cytol*. 1974;18:2–7.
- Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, Dondero F, Lenzi A. Study of apoptotic DNA fragmentation in human spermatozoa. *Hum Reprod*. 2000;15:830–839.
- Hoyes KP, Lord BI, McCann C, Hendry JH, Morris ID. Transgenerational effects of preconception paternal contamination with (55)Fe. *Radiat Res*. 2001;156:488–494.
- Jeyendran RS. *Interpretation of Semen Analysis Results: A Practical Guide*. Bath, United Kingdom: Cambridge University Press; 2000.
- Keel B, Webster BW. *Handbook of the Laboratory Diagnosis and Treatment of Infertility*. Boca Raton, Fla: CRC Press; 1990:4.
- Kocks MM, Muhring J, Knaapen MW, de Meyer GR. RNA synthesis and splicing interferes with DNA in situ end labeling techniques used to detect apoptosis. *Am J Pathol*. 1998;152:885–888.
- Koji T. Male germ cell death in mouse testes: possible involvement of Fas and Fas ligand. *Med Electron Microsc*. 2001;4:213–222.
- Kotwicka M, Jendraszak M, Warchol JB. Plasma membrane translocation of phosphatidylserine in human spermatozoa. *Folia Histochem Cytobiol*. 2002;40:111–112.
- Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S. Predictive value of abnormal sperm morphology in vitro fertilization. *Fertil Steril*. 1988;49:112–117.
- Lachaud C, Tesarik J, Canadas ML, Mendoza C. Apoptosis and necrosis in human ejaculated spermatozoa. *Human Reprod*. 2004;19:607–610.
- Lee J, Richburg JH, Younkin SC, Boekelheide K. The Fas system is a key regulator of germ cell apoptosis in the testis. *Endocrinology*. 1997;138:2081–2088.
- Levy R, Seifer-Akkin I. Apoptosis during spermatogenesis and in ejaculated spermatozoa: importance for fertilization. *Ann Biol Clin*. 2001;59:531–545.
- Martincic DS, Klun IV, Zorn B, Vrtovec HM. Germ cell apoptosis in the human testis. *Pflugers Arch*. 2001;442:R159–R160.
- Morris ID, Ilott S, Dixon L, Brison DR. The spectrum of DNA damage in human sperm assessed by single cell gel electrophoresis (Comet assay) and its relationship to fertilization and embryo development. *Hum Reprod*. 2002;17:990–998.
- Muratori M, Piomboni P, Baldi E, Filimberti E, Pecchioli P, Moretti E, Gambera L, Baccetti B, Biagiotti R, Forti G, Maggi M. Functional and ultrastructural features of DNA-fragmented human sperm. *J Androl*. 2000;21:903–912.
- Oehninger S, Morshedi M, Weng SL, Taylor S, Duran H, Beebe S. Presence and significance of somatic cell apoptosis markers in human ejaculated spermatozoa. *Reprod Biomed Online*. 2003;7:469–476.
- Oosterhuis GJ, Mulder AB, Kalsbeek-Batenburg E, Lambalk CB, Schoemaker J, Vermees I. Measuring apoptosis in human spermatozoa: a biological assay for semen quality? *Fertil Steril*. 2000;74:245–250.
- Paasch U, Grunewald S, Fitzl G, Glander HJ. Deterioration of plasma membrane is associated with activated caspases in human spermatozoa. *J Androl*. 2003;24:246–252.
- Print CG, Loveland KL. Germ cell suicide: new insights into apoptosis during spermatogenesis. *Bioessays*. 2000;22:423–430.
- Ricci G, Peticarari S, Fragonas E, Giolo E, Canova S, Pozzobon C, Guaschino S, Presani G. Apoptosis in human sperm: its correlation with semen quality and the presence of leukocytes. *Hum Reprod*. 2002;17:2665–2672.
- Roosen-Runge EC. Germinal-cell loss in normal metazoan spermatogenesis. *J Reprod Fertil*. 1973;35:339–348.
- Said TM, Paasch U, Glander HJ, Agarwal A. Role of caspases in male infertility. *Hum Reprod*. 2004;10:39–51.
- Sakkas D, Mariethoz E, St. John JC. Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. *Exp Cell Res*. 1999;251:350–355.
- Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N, Bizzaro

- D. Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. *Biol Reprod.* 2002;66:1061–1067.
- Shen HM, Dai J, Chia SE, Lim A, Ong CN. Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. *Hum Reprod.* 2002;17:1266–1273.
- Singh NP. A simple method for accurate estimation of apoptotic cells. *Exp Cell Res.* 2000;256:328–337.
- Singh NP, Muller CH, Berger RE. Effects of age on DNA double-strand breaks and apoptosis in human sperm. *Fertil Steril.* 2003;80:1420–1430.
- Sinha Hikim AP, Swerdloff RS. Hormonal and genetic control of germ cell apoptosis in the testis. *Rev Reprod.* 1999;4:38–47.
- Sun JG, Jurisicova A, Casper RF. Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization *in vitro*. *Biol Reprod.* 1997;56:602–607.
- Thonneau P, Marchand S, Tallec A, Ferial ML, Ducot B, Lansac J, Lopes P, Tabaste JM, Spira A. Incidence and main causes of infertility in a resident population (1850,000) of three French regions (1988–1989). *Hum Reprod.* 1991;6:811–816.
- Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. *J Immunol Methods.* 1995;184:39–51.
- Wang GR, Zhou ZD, Ge ZM, Zhao MJ. Preliminary investigation of relationship between sperm apoptosis and male infertility. *Zhonghua Nan Ke Xue.* 2002;8:25–27.
- Wang X, Sharma RK, Sikka SC, Thomas AJ Jr, Falcone T, Agarwal A. Oxidative stress is associated with increased apoptosis leading to spermatozoa DNA damage in patients with male factor infertility. *Fertil Steril.* 2003;80:531–535.
- Weil M, Jacobson MD, Raff MC. Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes. *J Cell Sci.* 1998;111:2707–2715.
- Weng SL, Taylor SL, Morshedi M, Schuffner A, Duran EH, Beebe S, Oehninger S. Caspase activity and apoptotic markers in ejaculated human sperm. *Mol Hum Reprod.* 2002;8:984–991.
- Williams GT, Smith CA. Molecular regulation of apoptosis: genetic control on cell death. *Cell.* 1993;74:777–779.
- World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction.* 4th ed. Cambridge, United Kingdom: Cambridge University Press; 1999.
- Wyllie AH, Kerr JF, Currie AR. Cell death: the significance of apoptosis. *Int Rev Cytol.* 1980; 68:251–306.