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Quantitative study of caspase-3 activity in semen and after swim-up preparation in relation to sperm quality

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BACKGROUND: There are no studies relating the apoptotic marker caspase-3 in human sperm to different degrees of abnormal sperm concentration, morphology and rapid progressive motility. METHODS: Semen from 67 males with abnormal semen analyses ($n = 61$) and normozoospermia ($n = 6$) were used. In each case, sperm from the neat semen (semen fraction) and after gradient centrifugation and swim-up (swim-up fraction) were incubated with a caspase-3 profluorogenic substrate. Caspase-3 activity was quantified in 119 850 sperm, 67 488 from semen and 52 362 from swim-up fractions. Logistic regression was used to estimate odds ratio (with 99% confidence intervals) for the presence of caspase-3-positive sperm. RESULTS: In semen fractions, no relationship was found between abnormal semen analysis subgroups and sperm caspase-3 activity. On the contrary, a significantly increased number of sperm with caspase-3 activity was found in the swim-up fractions from samples with poor sperm morphology. When analysis was restricted to single semen analysis defects, a significant increase of caspase-3-positive sperm was found in the semen fractions of cases with asthenozoospermia, and in the swim-up fractions of cases with teratozoospermia. CONCLUSIONS: Sperm caspase-3 activity seems to be associated with teratozoospermia and asthenozoospermia, thus suggesting that nuclear, mitochondrial and cytoskeletal abnormalities induce caspase-3 activation during spermiogenesis or sperm maturation.

Key words: apoptosis/asthenoteratozoospermia/caspase-3 activity/male infertility/semen analysis parameters

Introduction

Programmed cell death (apoptosis) is a predetermined event in the cell developmental programme, being involved in senescence as well as in the cell response to injuries. Apoptosis is under the control of caspases, cysteine proteinases that cleave key cellular proteins after aspartate residues. Caspases are resident in cells as inactive proenzymes and become activated by multiple mechanisms. Initiator caspases can be activated either by external signals (procaspases-2, -8 and -10) or by internal signals (procaspase-9) derived from mitochondria (cytochrome c). Both pathways trigger membrane phosphatidylserine translocation from the cytosolic to the external leaflet of the plasma membrane, thus conveying cells to phagocytosis. Crosstalk events also occur between these two main pathways, with caspase-8 being able to activate caspase-9 either directly or through mitochondrial and nuclear modulators. Both mechanisms are also capable of transducing p53 (proaptotic) nuclear stress signalling, and are modulated by Bcl2 (antiapoptotic) and Bax (proapoptotic). Effector caspases (procaspases-3, 6, 7) are then activated by initiator caspases, being responsible for cell proteolysis and DNA degradation through activation of an endonuclease that cleaves DNA between regularly spaced nucleosomal units (Scaffidi *et al.*, 1998; Slee *et al.*, 1999; Wolf *et al.*, 1999; Boatright *et al.*, 2003; Said *et al.*, 2004).

Apoptosis regulates normal spermatogenesis. In the postnatal period, it is responsible for the death and phagocytosis of premeiotic germ cells in order to adjust their number to the number of Sertoli cells, and for the removal of damaged and abnormal germ cells during active spermatogenesis in the post-pubertal period (Print and Loveland, 2000; Sinha Hikim *et al.*, 2003). Germ cell apoptosis has been shown to increase after testicular injury, such as exposure to toxics, varicocele, testicular torsion, hormonal deprivation and genetic abnormalities (Francavilla *et al.*, 2000; Kim *et al.*, 2001; Said *et al.*, 2004), as well as a response to freeze–thawing (Glander and Schaller, 1999; Schuffner *et al.*, 2001; Paasch *et al.*, 2004).

In the present investigation we quantitatively analysed caspase-3 activity in sperm retrieved from the semen and swim-up fractions of males with normal and abnormal semen parameters.

Materials and methods

Semen samples

This study was initiated after Institutional approval and patient informed consent. A total of 67 consecutive males undergoing spermiogram evaluation during infertility diagnosis were included. Criteria for inclusion were: (i) absence of known pathologies and intake of medicines; (ii) normal physical examination, hormonal profiles and karyotypes; (iii) spermiogram analysis showing presence of sperm, normal pH, absence of agglutination and negative microorganism culture. The mean age was 34.9 ± 4.88 years SD (range 27–52), with 88.1% aged <39 years (age groups: 25–29 years, $n = 6$; 30–34 years, $n = 30$; 35–39 years, $n = 23$; 40–44 years, $n = 5$; 45–49 years, $n = 2$; 50–54 years, $n = 1$). Each patient contributed a single semen sample. Each semen sample was left to liquefy at 37°C for 30 min; a small fraction was taken for caspase-3 activity determination (semen fraction) and another aliquot was used for spermiogram analysis (World Health Organization, 1999). The rest of the semen was submitted to gradient centrifugation at 437 *g* for 20 min at room temperature (Ixaprep; Medicult, Denmark), to select sperm with normal morphology and motility at the bottom of the tubes. The pellet was washed (194 *g* for 2 × 10 min at room temperature) in sperm preparation medium (SPM, with HEPES buffer; Medicult), layered with 50–100 μl of SPM, and incubated (1 h, 37°C, 5% CO_2 in humidified air) to collect the swim-up fraction containing motile normal sperm that actively migrated from the pellet to the upper aqueous phase. This fraction was then taken for determination of caspase-3 activity in the swim-up. Seven cases were submitted to the swim-up technique only: three with oligoteratozoospermia (0.16–0.6 × 10^6/ml; 25–33.3% rapid progressive motility; 0% of normal morphology but with insufficient sperm for rigorous quantitative normal morphology assessment) and four with oligoasthenoteratozoospermia (0.05–1.8 × 10^6/ml; 3.3–13.5% rapid progressive motility; 0% normal morphology but with insufficient sperm for morphology assessment in three cases).

Caspase-3 activity detection

Fractions used for caspase-3 activity determination were centrifuged at 1300 *g* for 2 min at room temperature. The pellet was resuspended and incubated for 30 min (37°C, 5% CO_2 in humidified air) in previously warmed 50 μl of medium containing (1:1) SPM and PhiPhiLux D2G2 (membrane permeant profluorogenic caspase-3 substrate; OncoImmunin Inc., USA; stock solution: 10 μmol/l in Roswell Park Memorial Institute 1640 medium with 25 mmol/l HEPES, kept in aliquots at −20°C). Semen and swim-up sperm fractions were then washed (1300 *g* for 2 × 1 min at room temperature) with 500 μl of phosphate-buffered saline (Sigma, Barcelone, Spain), and the pellet spread onto poly-L-lysine (Sigma)-coated glass slides. After air-drying, slides were mounted with 10 μl Vectashield antifade medium (Vector Laboratories, USA), containing 1.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) to stain the DNA (blue), and sperm counted in an epifluorescence microscope (Eclipse, E-400; Nikon, Japan) fitted with a CCD camera (Sony, Japan) and an automated karyotyper software (Cytovision Ultra; Applied Imaging International, UK). In the presence of activated caspase-3, the specific substrate PhiPhiLux G2D2 is cleaved and emits red light. In all cases, sperm positive for caspase-3 activity showed an intense fluorescent red colour in the mitochondrial midpiece (Figure 1).

About 1000 sperm were counted (double-blind procedure) per semen and per swim-up fractions of each individual. The rate of caspase-3-positive sperm was expressed as the number of caspase-3-positive sperm per 100 sperm. In 25 cases, <1000 sperm could be counted (mean: 377.14 sperm per fraction, range: 22–986). Of these, three cases had insufficient sperm in both semen and swim-up fractions, corresponding to two patients with oligoasthenoteratozoospermia (0.05–0.26 × 10^6/ml; 3.3–5.4% rapid progressive motility; 0% normal morphology but with insufficient sperm for rigorous quantitative normal morphology assessment) and one patient with oligoteratozoospermia (0.16 × 10^6/ml; 24.8% rapid progressive motility; 0% normal morphology but with insufficient sperm for morphology assessment). In the other 22 cases, there was insufficient sperm in the swim-up fraction: two patients with teratozoospermia (40–170 × 10^6/ml; 44.4–46.5% rapid progressive motility; 6–11% normal morphology), two with asthenoteratozoospermia (20–75 × 10^6/ml; 0.4–1.8% rapid progressive motility; 0–5% normal morphology), seven with oligoteratozoospermia (0.36–14 × 10^6/ml; 25.6–59.3% rapid progressive motility; 0–5% normal morphology but with insufficient sperm for morphology assessment in one case), and 11 with oligoasthenoteratozoospermia (0.17–18.5 × 10^6/ml; 1–20.4% rapid progressive motility; 0–11% normal morphology but with insufficient sperm for morphology assessment in one case).

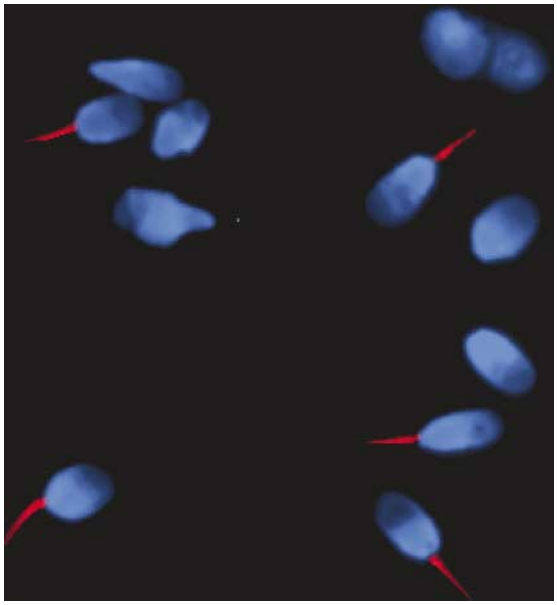

Figure 1. Epifluorescence image of sperm from a semen fraction of a patient with oligoasthenoteratozoospermia. Caspase-3 activity (red) appears restricted to the sperm mitochondrial midpiece. DNA is stained in blue with DAPI.

Statistical analysis

Descriptive statistics were used for the initial characterization of the population, considering each male as an individual and a two-sided significance level of 5%. In this approach, Wilcoxon and Kruskal–Wallis tests were used to compare the percentages of caspase-3-positive sperm between semen and swim-up fractions, as well as between the defined levels of each parameter in each fraction. This approach is considered the traditional way for analysing this kind of outcome, where a number of cells are observed for each subject and the result is represented as a percentage of positive cells. A limitation of this traditional approach is that the total number of cells counted is not considered, and the characteristics observed for each cell is addressed only to the subject to which it belongs. Thus, data were then analysed at the cellular level, by which each spermatozoon scored was considered independently from other cells (Zhao *et al.*, 2001). In this approach, the cells are considered as

the individuals, and for each one the outcome, caspase-3-positive or caspase-3 negative sperm, corresponds to a binary variable. When data were analysed at the cellular level, the size of the sample studied and the power of the hypothesis tested were greatly increased. To control the possibility of committing a type I error, hypotheses were tested at a probability of 0.01 rather than 0.05, and thus a stringent significance level of 1% was chosen for statistical significance. The presence or absence of positive caspase-3 sperm was studied with logistic regression models and the odds ratio (OR) for risk of presence and their associated 99% confidence interval (CI) are presented. Firstly, a comparative analysis between semen and swim-up fractions was performed. To find any possible association between the presence of caspase-3 and the different spermiogram parameters, semen and swim-up fractions were then analysed separately. All analyses were performed with the SPSS statistical package (version 11.0) for Windows.

Results

Two types of control groups were used: relative controls, specific for each spermiogram parameter ($\geq 20 \times 10^6$/ml sperm concentration; or $\geq 15\%$ normal morphology; or $\geq 25\%$ rapid progressive motility), and absolute controls, derived from samples showing all spermiogram parameters simultaneously normal (normozoospermia: $\geq 20 \times 10^6$/ml sperm concentration + $\geq 15\%$ normal morphology + $\geq 25\%$ rapid progressive motility). Three study groups were used, based on the three main spermiogram parameters. Each group was further divided into different subgroups of severity (Table I). A different study group classification was also made with dichotomous variables; an individual was classified based on single spermiogram defects into oligo-, terato- and asthenozoospermia. In this case, only one semen parameter was affected in each individual. In the present study, there were no patients with pure oligozoospermia. When patients showed more than one affected semen parameter, they were classified into other groups, oligoastheno-, oligoterato-, asthenoterato- or oligoasthenoteratozoospermia (Table I). The number of cases and sperm analysed, as well as the means ± SD of caspase-3-positive sperm in all control and study groups, either in the semen and swim-up fractions, are shown in Table I.

The comparison of the presence of caspase-3-positive sperm between semen and swim-up fractions, considering both the males and the cells as individuals, revealed significant differences ($P < 0.001$), with semen fractions showing

Table I. Caspase-3-positive sperm in semen fractions and swim-up fractions per subgroup analysed

Groups/subgroups	Cases	Semen fraction			Swim-up fraction		
		Sperm	Caspase-3$^+$ sperm		Sperm	Caspase-3$^+$ sperm	
	(*n*)	(*n*)	(*n*)	(%)[a]	(*n*)	(*n*)	(%)[a]
Concentration ($\times 10^6$/ml)							
≤1	6	4237	605	14.3 ± 6.6	1501	40	2.7 ± 4.9
> 1– ≤ 5	6	6129	775	12.6 ± 4.7	4349	96	2.2 ± 0.7
> 5– < 10	11	11 215	1742	15.5 ± 6.3	6576	189	2.9 ± 2.0
≥ 10– < 20	10	10 550	1175	11.1 ± 8.3	6859	199	2.9 ± 2.8
<20	33	32 131	4297	13.4 ± 7.1	19285	524	2.7 ± 2.5
≥20	34	35 357	6560	18.6 ± 9.2	33 077	1748	5.3 ± 3.5
% normal morphology							
≤5	28	28 817	3773	13.1 ± 6.2	18 580	606	3.3 ± 2.4
> 5– < 10	13	13 621	2600	19.1 ± 5.5	12 778	636	5.0 ± 4.0
≥ 10– < 15	11	11 509	1895	16.5 ± 8.3	10 261	649	6.3 ± 3.3
<15	52	53 947	8268	15.3 ± 7.0	41 619	1891	4.5 ± 3.5
≥15	9	9304	1984	21.3 ± 14.2	9242	341	3.7 ± 2.3
% rapid progressive motility							
≤5	7	6359	863	13.6 ± 7.6	3540	173	4.9 ± 2.5
> 5– < 10	5	5066	818	16.1 ± 6.7	2532	188	7.4 ± 2.3
≥ 10– < 25	22	21 910	3302	15.1 ± 8.1	17 685	606	3.4 ± 2.9
<25	34	33 335	4983	15.0 ± 7.8	23 757	967	4.1 ± 3.1
≥25	33	34 153	5874	17.2 ± 9.2	28 605	1305	4.6 ± 3.6
Absolute control	6	6222	1272	20.4 ± 15.5	6137	234	3.8 ± 2.7
Asthenozoospermia	2	2054	524	25.5 ± 12.8	2088	79	3.8 ± 1.6
relative control	65	65 434	10 333	15.8 ± 8.3	50 274	2193	4.4 ± 3.4
Teratozoospermia	17	17 775	3064	17.2 ± 6.2	16 511	941	5.7 ± 3.7
relative control	50	49 713	7793	15.7 ± 9.3	35 851	1331	3.7 ± 3.0
Oligoasthenozoospermia	1	1028	188	18.3	1017	28	2.8
relative control	62	64 252	10 429	16.2 ± 8.7	50 967	2206	4.8 ± 3.4
Oligoteratozoospermia	8	8127	1173	14.4 ± 8.2	4834	128	2.6 ± 2.3
relative control	58	57 193	9309	16.3 ± 8.8	46 293	2137	4.6 ± 3.4
Asthenoteratozoospermia	9	9306	1700	18.3 ± 5.9	8341	494	5.9 ± 3.3
relative control	58	56 053	8932	15.9 ± 9.0	43 670	1743	4.0 ± 3.2
Oligoasthenoteratozoospermia	18	18 739	2331	12.4 ± 6.7	11 933	328	2.7 ± 2.2
relative control	45	46 541	8286	17.8 ± 8.9	40 051	1906	4.8 ± 3.5
Total	67	67 488	10 857	16.1 ± 8.6	52 362	2272	4.7 ± 3.9

[a]Mean ± SD.
Absolute control: $\geq 20 \times 10^6$/ml concentration + ≥ 15% normal morphology + ≥ 25% rapid progressive motility.
Relative control: all other individuals of the population regarding each considered subgroup.

4.2-fold more caspase-3 activity than swim-up fractions (OR = 4.227, 99% CI = 3.975–4.495). In order to obtain a better knowledge of these differences and to investigate the possible association between the presence of caspase-3 activity and sperm concentration, normal morphology and rapid progressive motility, the two sperm fractions were analysed separately.

Semen fractions

Considering each male as an individual, the percentage values of caspase-3-positive sperm appeared much dispersed in the different groups of spermiogram parameters, thus suggesting that no relationship exists (Figure 2); nor were there significant differences with respect to subgroups ($P \geq 0.070$) for sperm concentration, normal morphology or rapid progressive motility.

Data were then treated at the cellular level, in which the individual was each spermatozoon scored. The presence or absence of caspase-3-positive sperm was analysed using logistic regression and the odds ratio calculated for each variable, with the reference category being the one referred to the normal value (relative control) of the parameter under analysis (Table II). All main spermiogram parameters were significantly associated with the presence of caspase-3-positive sperm ($P < 0.001$). The probability of the spermatozoon being caspase-3 positive was significantly lower ($P < 0.001$) than the reference category (relative control) in all subgroups, except for the $> 5- < 10\%$ rapid progressive motility subgroup, where no significant differences were found ($P = 0.063$). To further consolidate these results, a similar analysis was performed with a more strict reference group (absolute control), where all the spermiogram parameters were normal (Table II). Again, all main spermiogram parameters were significantly associated with the presence of caspase-3-positive sperm ($P < 0.001$), with higher risk in the reference category (absolute control), except for the $> 5- < 10\%$ normal morphology subgroup where no significant differences were found ($P = 0.025$). To uncover the individual contribution of each abnormal spermiogram parameter to these results, dichotomous study groups were then analysed (Table III). Taking all other individuals of the population as control, there were significant differences in all cases with the exception of the oligoasthenozoospermic group ($P = 0.077$), with a higher risk for the presence of caspase-3-positive sperm being demonstrated in the astheno-, terato- and asthenoteratozoospermic subgroups (OR ≥ 1, $P < 0.001$). When groups were compared to the absolute control, the oligoasthenozoospermic group again showed no significant differences ($P = 0.111$) and only the asthenozoospermic group showed significantly increased numbers of caspase-3-positive sperm (OR = 1.333, $P < 0.001$).

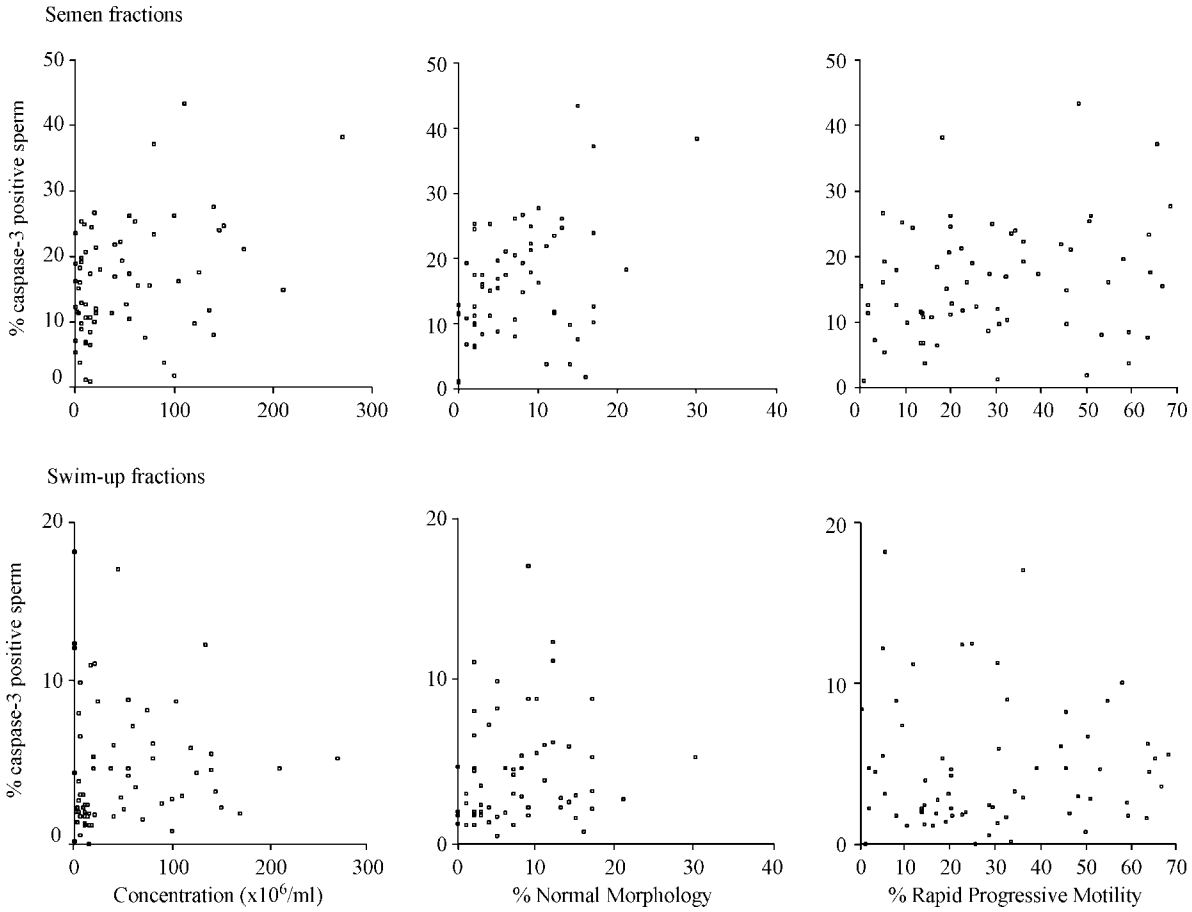

Figure 2. Scatters of the percentage of caspase-3-positive sperm in the semen fractions and in the swim-up fractions, among the different spermiogram parameters.

Table II. Odds ratio for risk of presence of caspase-3-positive sperm in semen and swim-up fractions per subgroup analysed

Groups/ subgroups	Semen fraction				Swim-up fraction			
	I		II		I		II	
	OR (99% CI)	*P*	OR (99% CI)	*P*	OR (99% CI)	*P*	OR (99% CI)	*P*
Concentration ($\times 10^6$/ml)		< 0.001		< 0.001		< 0.001		< 0.001
≤ 1	0.731 (0.650–0.823)	< 0.001	0.648 (0.564–0.745)	< 0.001	0.491 (0.323–0.745)	< 0.001	0.691 (0.442–1.080)	0.033
> 1– ≤ 5	0.635 (0.572–0.706)	< 0.001	0.563 (0.496–0.640)	< 0.001	0.405 (0.308–0.532)	< 0.001	0.570 (0.415–0.782)	< 0.001
> 5– < 10	0.807 (0.748–0.871)	< 0.001	0.716 (0.644–0.795)	< 0.001	0.530 (0.434–0.648)	< 0.001	0.747 (0.578–0.965)	0.003
≥ 10– < 20	0.550 (0.504–0.600)	< 0.001	0.488 (0.435–0.546)	< 0.001	0.534 (0.439–0.650)	< 0.001	0.752 (0.584–0.968)	0.004
≥ 20	0.678 (0.641–0.716)	< 0.001	0.601 (0.548–0.658)	< 0.001	0.500 (0.439–0.570)	< 0.001	0.704 (0.573–0.865)	< 0.001
% normal morphology		< 0.001		< 0.001		< 0.001		< 0.001
≤ 5	0.556 (0.514–0.602)	< 0.001	0.586 (0.534–0.643)	< 0.001	0.879 (0.736–1.050)	0.062	0.850 (0.694–1.040)	0.038
> 5– < 10	0.870 (0.799–0.949)	< 0.001	0.918 (0.832–1.013)	0.025	1.367 (1.146–1.631)	< 0.001	1.321 (1.081–1.616)	< 0.001
≥ 10– < 25	0.727 (0.663–0.797)	< 0.001	0.767 (0.692–0.851)	< 0.001	1.762 (1.477–2.102)	< 0.001	1.703 (1.393–2.082)	< 0.001
≥15	0.668 (0.621–0.718)	< 0.001	0.704 (0.646–0.768)	< 0.001	1.242 (1.064–1.450)	< 0.001	1.200 (1.001–1.440)	0.010
% rapid progressive motility		< 0.001		< 0.001		< 0.001		< 0.001
≤ 5	0.756 (0.683–0.837)	< 0.001	0.611 (0.540–0.692)	< 0.001	1.072 (0.865–1.327)	0.404	1.292 (1.992–1.684)	0.012
> 5– < 10	0.927 (0.835–1.030)	0.063	0.749 (0.660–0.851)	< 0.001	1.678 (1.362–2.067)	< 0.001	2.023 (1.560–2.624)	< 0.001
≥ 10– < 25	0.854 (0.804–0.908)	< 0.001	0.691 (0.628–0.759)	< 0.001	0.742 (0.652–0.845)	< 0.001	0.895 (0.731–1.096)	0.158
≥ 25	0.846 (0.802–0.893)	< 0.001	0.684 (0.625–0.748)	< 0.001	0.887 (0.793–0.992)	0.006	1.070 (0.883–1.295)	0.364

OR (99% CI) = odds ratio (99% confidence interval). $P < 0.01$ is considered significant.
Reference categories: (I) relative control groups for each spermiogram parameter; (II) absolute control group.
Relative control groups: ≥20 × 10^6/ml concentration; ≥15% normal morphology; ≥25% rapid progressive motility.
Absolute control group: ≥20 × 10^6/ml concentration + ≥15% normal morphology + ≥25% rapid progressive motility.

Table III. Odds ratio for risk of presence of caspase-3-positive sperm in semen fractions and swim-up fractions per subgroup analysed

Subgroups	Semen fraction				Swim-up fraction			
	I		II		I		II	
	OR (99% CI)	*P*	OR (99% CI)	*P*	OR (99% CI)	*P*	OR (99% CI)	*P*
A	1.826 (1.598–2.087)	< 0.001	1.333 (1.143–1.554)	< 0.001	0.862 (0.638–1.165)	0.205	0.992 (0.705–1.396)	0.952
T	1.120 (1.055–1.190)	< 0.001	0.811 (0.737–0.892)	< 0.001	1.568 (1.401–1.755)	< 0.001	1.525 (1.285–1.848)	< 0.001
OA	1.155 (0.936–1.424)	0.077	0.871 (0.697–1.089)	0.111	0.626 (0.381–1.029)	0.015	0.714 (0.424–1.205)	0.097
OT	0.868 (0.796–0.946)	< 0.001	0.656 (0.585–0.736)	< 0.001	0.562 (0.443–0.713)	< 0.001	0.686 (0.515–0.915)	0.001
AT	1.179 (1.093–1.271)	< 0.001	0.870 (0.782–0.968)	0.001	1.515 (1.323–1.734)	< 0.001	1.588 (1.288–1.958)	< 0.001
OAT	0.656 (0.615–0.700)	< 0.001	0.553 (0.501–0.610)	< 0.001	0.565 (0.483–0.661)	< 0.001	0.712 (0.569–0.891)	< 0.001

OR (99% CI): odds ratio (99% confidence interval). $P < 0.01$ is considered significant.
Reference categories: (I) all other individuals of the population or (II) absolute control group (≥20 × 10^6/ml concentration + ≥15% normal morphology + ≥25% rapid progressive motility).
O = oligozoospermia; A = asthenozoospermia; T = teratozoospermia.

Swim-up fractions

As observed for the semen fractions, when considering each male as an individual the percentage values of caspase-3-positive sperm appeared quite dispersed in the different groups of spermiogram parameters (Figure 2). With respect to subgroups, no significant differences were found ($P \geq 0.066$) for sperm concentration, rapid progressive motility or normal morphology.

When data were treated at the cellular level using comparisons to the relative controls, all main spermiogram parameters (concentration, normal morphology, rapid progressive motility) appeared significantly associated ($P < 0.001$) with the presence of caspase-3-positive sperm (Table II). No significant differences from the relative controls were found in the ≤5% subgroups of normal morphology ($P = 0.062$) and rapid progressive motility ($P = 0.404$). The probability of the spermatozoon being caspase-3-positive was significantly lower than the reference category in all other subgroups, except for a significant increase in the proportion of caspase-3-positive sperm relative to controls in the subgroups > 5– < 10%, ≥ 10– < 15% and <15% of normal morphology, and > 5– < 10% of rapid progressive motility (OR ≥ 1, $P < 0.001$). To further consolidate the present results, a similar analysis was performed using comparisons to the absolute control group (Table II). In this case, significant differences ($P < 0.001$) were found for the presence of caspase-3-positive sperm regarding all main spermiogram parameters. No significant differences from the absolute control

were found in the $\leq 1\%$ subgroup of sperm concentration ($P = 0.033$), in the $\leq 5\%$ ($P = 0.038$) and $< 15\%$ ($P = 0.010$) subgroups of normal morphology, and in the $\leq 5\%$ ($P = 0.012$), $\geq 10 - < 25\%$ ($P = 0.158$) and $< 25\%$ ($P = 0.364$) subgroups of rapid progressive motility. With the exception of a significant increase in the proportion of caspase-3-positive sperm relative to the control in the subgroups $> 5 - < 10\%$ and $\geq 10 - < 15$; of normal morphology, and $> 5 - < 10\%$ of rapid progressive motility ($OR \geq 1$, $P < 0.001$), the probability of the spermatozoon being caspase-3 positive was significantly lower than the reference category in all other subgroups. In the analysis of dichotomous study groups (Table III), comparisons with the relative controls or the absolute control showed significant differences in all subgroups, with the exception of the asthenо- and oligoasthenozoospermic subgroups ($P > 0.01$). This also demonstrated a higher risk for the presence of caspase-3-positive sperm in the terato- and asthenoteratozoospermic subgroups ($OR \geq 1$, $P < 0.001$).

Discussion

Normal human spermatogenesis is modulated by apoptotic events that adjust the number of germ cells to the number of Sertoli cells and eliminate the abnormal gametes. Genetic and exogenous injuries influencing spermatogenesis potentiate the endogenous mechanism of germ cell apoptosis, and may lead to insufficient or absent sperm production. We show here that the presence of the major effector caspase-3 is related to asthenozoospermia and teratozoospermia.

Although it remains to be established whether sperm defects are a cause or a consequence of increased rates of apoptosis, several studies have shown an increase of DNA fragmentation in human sperm with low motility (Barroso *et al.*, 2000; Weng *et al.*, 2002), low sperm counts (Oosterhuis *et al.*, 2000) or decreased normal sperm concentration, morphology and motility (Gandini *et al.*, 2000; Shen *et al.*, 2002). This is of clinical relevance, as semen samples showing increased rates of sperm DNA fragmentation have been associated with decreased rates of fertilization (Marchetti *et al.*, 2002) and pregnancy (Tomlinson *et al.*, 2001). Other studies have also shown that mitochondrial integrity is decreased in samples with low sperm motility (Donnelly *et al.*, 2000; Weng *et al.*, 2002), and that phosphatidylserine exposure is increased in samples exhibiting low sperm motility (Weng *et al.*, 2002), decreased normal sperm motility and morphology (Shen *et al.*, 2002), or decreased normal sperm concentration and motility (Oosterhuis *et al.*, 2000).

Here we present a strict statistical analysis of 67 semen samples, in which 119 850 sperm were quantified for the presence of caspase-3 activity, 67 488 from semen fractions and 52 362 from swim-up fractions. The large number of cases studied enabled the analysis of different subgroups of severity of sperm defects within major groups of spermiogram abnormalities. In the first approach, cases were divided according to decreased sperm normal concentration, morphology and rapid progressive motility, and each group was then subdivided into different levels of severity of each sperm defect. In a second approach, and to uncover the possible contribution of simultaneous sperm defects, cases were analysed for single abnormalities (oligo-, astheno- and teratozoospermia). In the semen fractions, no relationship was found between the presence of caspase-3-positive sperm and any of the abnormal spermiogram values. In accordance with previous results (Ricci *et al.*, 2002), the proportion of caspase-3-positive sperm was shown to be unrelated to sperm quality. However, we first show that this is due to the presence of simultaneous sperm defects, as the analysis of single sperm abnormalities revealed a strong relationship to asthenozoospermia. On the contrary, results obtained from the swim-up fractions showed a significant increase of caspase-3-positive sperm with decreased normal morphology and rapid progressive motility values, which after analysis of single sperm abnormalities was only confirmed for teratozoospermia. In accordance with previous reports that have used several other markers for detecting sperm apoptosis (Donnelly *et al.*, 2000; Gandini *et al.*, 2000; Marchetti *et al.*, 2002), our current results confirm that gradient centrifugation and swim-up significantly deplete caspase-3-positive sperm. Taking into account that the presence of caspase-3-positive sperm was 4.2-fold higher in semen than in the swim-up fractions ($OR = 4.227$, 99% $CI = 3.975 - 4.495$), that the swim-up fractions were associated only with teratozoospermia ($OR \geq 1$ and $P < 0.001$), and that samples with decreased normal sperm morphology ($< 15\%$) have $4.5 \pm 3.5\%$ (mean $\pm$ SD) of caspase-3-positive sperm in the swim-up fractions, the data suggest a low risk of selecting apoptotic sperm during clinical treatments.

Caspase activation is a well-defined point of no return for apoptotic progression in somatic cells (Wolf *et al.*, 1999). Although procaspases are present in human mature sperm and might be activated by different stimuli such as cryopreservation (Paasch *et al.*, 2004), the role of caspases and apoptosis in ejaculated sperm is still an open question. Caspase activity in mature sperm may correspond to the activation of the apoptotic machinery leading to cell death as induced by different factors during spermiogenesis. In this mechanism, spermatids marked for elimination via apoptosis could somehow escape the removal mechanism and thus contribute to the poor sperm quality found in the ejaculate (Sakkas *et al.*, 2002; Said *et al.*, 2004). However, as active caspase-1 was demonstrated to be involved in the degradation of surplus spermatid cytoplasmic components (residual bodies) during spermiogenesis, caspase activity in ejaculated sperm may, alternatively, correspond to enzyme diffusion from residual bodies (Blanco-Rodriguez and Martinez-Garcia, 1999). Caspase-3 is the major effector enzyme causing cell disruption during apoptosis. Caspase-3 activity has been previously detected in the midpiece of ejaculated human sperm (Weng *et al.*, 2002) and shown to be significantly associated with low sperm motility (Weng *et al.*, 2002) or with decreased normal sperm concentration, motility and morphology (Wang *et al.*, 2003). Our present results confirm that sperm caspase-3 activity is restricted to the mitochondrial sheath, and show

no statistical association between the presence of caspase-3-positive sperm and decreased normal sperm concentration, motility or morphology in the neat semen. On the contrary, a significant increase of caspase-3-positive sperm is shown in the swim-up fractions from samples with decreased normal sperm morphology. These data also show that when the analysis is corrected for single sperm defects, caspase-3-positive sperm appear significantly associated with asthenozoospermia in the semen fractions and with teratozoospermia in the swim-up fractions. These data thus strongly favour the hypothesis that caspase activity is not the result of active enzyme diffusion from residual bodies and may correspond to the activation of the cell apoptotic machinery for discarding abnormal spermatids/sperm due to nuclear, mitochondrial and cytoskeleton structural defects.

In conclusion, no relationship was found between caspase-3-positive sperm and abnormal spermiogram subgroups in the semen fractions, whereas a significant relationship was found between caspase-3-positive sperm and decreased normal sperm morphology and rapid progressive motility in the swim-up fractions. When the analysis was restricted to single spermiogram defects, a significant increase in caspase-3-positive sperm was found in relation to asthenozoospermia in the semen and to teratozoospermia in the swim-up fraction.

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