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ORIGINAL ARTICLE

Relationship between apoptotic markers in semen from fertile men and demographic, hormonal and seminal characteristics

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Apoptosis in the testis has two putative roles during normal spermatogenesis; limitation of the germ cell population to numbers that can be supported by the Sertoli cells, and, possibly, selective depletion of meiotic and postmeiotic abnormal germ cells. We investigated the demographic and biological correlates of the pro-apoptotic marker Fas and the anti-apoptotic marker Bcl-xL in sperm cells of fertile men. Six hundred and four men from Greenland, Poland and Ukraine were consecutively enrolled during their pregnant wife's antenatal visits. Semen analysis was performed as recommended by the World Health Organization. Immunofluorescence coupled to flow cytometry was utilized for detection of apoptotic markers in the sperm cell. DNA damage was assessed by flow cytometry using both the sperm chromatin structure assay (SCSA) and the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay. The percentage of Fas-positive sperm cells was higher in men with high total sperm count ($P<0.01$), more motile sperms ($P=0.04$) and fewer sperm head defects ($P=0.05$). These associations were consistent within and across study regions. Furthermore, testosterone, follicle-stimulating hormone (FSH) and sexual hormone-binding globulin (SHBG) were significantly negatively correlated with Fas within and across regions as well. The data indicated no association between the anti-apoptotic Bcl-xL marker and semen or personal characteristics. The finding of Fas-positive sperm cells associated with better semen quality in a cohort of spouses of pregnant women seems different from previous data obtained in infertile men and warrants further investigation to clarify the biological significance of sperm apoptotic markers.
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INTRODUCTION

Apoptosis is the underlying mechanism of programmed death of germ cells during human spermatogenesis and is crucial for testis homeostasis during the whole reproductive lifetime. Like other cell types, male germ cells respond to external and internal stimuli by intracellular signalling pathways that ultimately determine their fate.[1] Proteins of the Bcl-2 family provide one signalling pathway with some members promoting and other members antagonizing cell death.[1,2] Fas/FasL provides another pathway for germ cell apoptosis.[3] Apoptotic pathways converge at the level of caspases, which are considered to be the key enzymes of apoptosis, acting both as initiators and executers of programmed cell death,[1,3] but caspase independent pathways have also been described.[4] The finely-tuned apoptotic mechanisms eliminate abnormal cells, and thereby minimize the negative consequences of abnormal spermatozoa on male fertility and on the health of offspring.

In human semen samples, a variable fraction of sperm cells is present with DNA strand breaks. Several theories have been put forward to explain the existence of these abnormal cells,[5–7] present also in otherwise normal ejaculates as assessed by the World Health Organisation's (WHO) guidelines for conventional semen analysis (WHO, 1999). It has been proposed that sperm DNA damage can originate from oxidative stress, from unligated strand breaks physiologically formed during spermatogenesis to help the process of chromatin remodelling, and from defective or 'abortive' apoptotic processes[8–10] initiated post meiotically when cells are no longer able to complete the classical pathways of programmed cell death.[1,3,11] Notably, it has been stressed that these mechanisms are not mutually exclusive and, in reality, DNA damage may arise from combinations of all three mechanisms.[5]

The theoretical framework of abortive apoptosis is based on the assumption that as germ cells progress into their terminal differentiation pathway, they become more and more transcriptionally and translationally silent and progressively lose their capacity to undergo programmed cell death in the form of classical apoptosis. Instead, a restricted form of apoptosis takes over, i.e., a sort of regulated cell

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death,[5] mirrored by the presence of nuclear DNA strand breaks in human ejaculated spermatozoa (as assessed by terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay) and abnormal persistence of some proteins activated during the extrinsic and intrinsic apoptotic pathways,[8,12] such as the pro-apoptotic marker Fas and the anti-apoptotic marker Bcl-xL. In addition, other apoptotic hallmarks, such as caspase activation and phosphatidylserine exteriorization, have been detected in human spermatozoa.[13] Intriguingly, from studies where a variety of apoptotic-like sperm biomarkers were measured and correlated, it emerged that TUNEL-positive spermatozoa (which are spermatozoa with DNA breaks highlighted by labelling 3′-OH termini) and Fas, Bcl-x and p53 expression did not always exist in unison, but semen samples that had a low sperm concentration and poor morphology were more likely to show high levels of TUNEL positivity and expression of Fas and p53.[14] Even though the occurrence of this complex pattern of apoptotic-like earmarks in human sperm is far from being completely understood, the expression of sperm apoptotic markers has mainly been studied in men with infertility problems in comparison with a variety of control subjects. Limited knowledge is available from fertile population-based studies and no demographic factors influencing sperm cell apoptosis have been analysed. In this connection, applications in the field of molecular epidemiology were attempted under the hypothesis that exposures to environmental stressors could exacerbate the abnormal expression of these proteins in ejaculated human sperm.[15–17]

The aim of the present study was to examine demographic, hormonal and seminal correlates of the expression of the pro-apoptotic marker Fas and the anti-apoptotic marker Bcl-xL in sperm from spouses of pregnant women. We choose to analyse Fas and Bcl-xL rather than other seminal markers as caspase-3, caspase-8, caspase-9 and phosphatidylserine externalization, because the former have been associated with male infertility in a few earlier studies of infertility clients.[7,8] A large-scale population-based study of correlates of Fas and Bcl-xL has never been performed.

MATERIALS AND METHODS

For this cross-sectional semen study we collected semen and blood samples between May 2002 and February 2004 from 604 men with pregnant spouses in the three study regions: Greenland, Poland and Ukraine as described previously.[18]

Study population

In the original study, altogether 3833 pregnant women were encouraged to participate consecutively at their first antenatal care visit at three locations: (i) local hospitals in 19 cities and settlements throughout Greenland; (ii) a large central hospital in Warsaw, Poland; and (iii) three hospitals and eight antenatal clinics in Kharkiv, Ukraine.

In all countries, it was required that both partners were 18 years or more of age and born in the country of the study. The participation rate among all women who were invited to participate was 26% (640/2478) in Kharkiv, 68% (472/690) in Warsaw and 90% (598/665) in Greenland.[15] In total 1710 pregnant women (45%) were included in the study.

The male partners in this semen study were enrolled until a total of approximately 200 men had been reached in each region.[19] The male partners were interviewed regarding lifestyle, occupational and reproductive history. The interview included questions regarding occupational factors, urogenital disorders, sexual abstinence and issues regarding delivery of a semen sample. Information about smoking habits, diet (seafood intake, caffeinated drinks and alcohol consumption) was obtained as well, but with reference to the period when the couple tried to become pregnant. Further details are described by Toft *et al.*[18]

The local ethical committees representing all participating populations approved the study and each subject signed an informed consent.

Collection of semen and blood samples

Participants were instructed to collect a semen sample by masturbation at their residence or in privacy in a room at the hospital after at least 2 days of sexual abstinence. Semen was collected in a sterile plastic container provide by the project team. The actual period of sexual abstinence was recorded. The sample was kept close to the body to maintain a temperature close to 37°C when transported to the laboratory immediately after collection. After liquefaction, and no longer than 1 h after ejaculation, semen samples were analyzed for concentration of spermatozoa, according to recommendations by the WHO[19] and motility was analyzed according to computer-assisted sperm analysis in laboratories in the three study regions. The morphology of the sperm from the three study regions was determined centrally by two technicians at the Fertility Centre, Malmö University Hospital, on Papanicolaou-stained smears using the WHO (1999) criteria. Aliquots of the assessment of DNA damage, the expression of apoptotic markers and reproductive hormones were immediately frozen and stored at −80°C.

The blood samples were centrifuged immediately after collection and sera were stored at −80°C for later analysis.[19]

Determination of reproductive hormones

Serum concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH) and estradiol were analyzed with immunofluorometric techniques. The total assay variation coefficients were 2.9%, 2.6% and 8.1%, respectively. Serum testosterone and sexual hormone-binding globulin (SHBG) were measured by commercially available immunoassays. The total assay variation coefficients were 5.5% and 4.6%, respectively. Inhibin B levels were assessed using a specific immunometric method, as previously described, with a detection limit of 15 ng l^{-1} and intra-assay and total assay variation coefficients <7%.[20,21]

All assays were performed at Malmö University Hospital (Sweden) after completion of sample collection.

Determination of sperm DNA integrity

Sperm chromatin structure assay (SCSA). Coded frozen semen samples from the three study regions were shipped on dry ice to the flow cytometry (FCM) facility of the Section of Toxicology and Biomedical Sciences (ENEA Casaccia, Rome, Italy) for SCSA analysis carried out following strictly the standardized procedure described by Evenson *et al.*[22] Briefly, SCSA is a FCM technique that identifies the spermatozoa with abnormal chromatin packaging and/or with DNA breaks by an increased susceptibility to acid induced DNA denaturation *in situ*. Acridine orange (Molecular Probes, Eugene, OR, USA) staining is used to distinguish between denaturated (red fluorescence, single-stranded) and native (green fluorescence, double-stranded) DNA regions in sperm chromatin. Cells were analysed by a FACScan flow cytometer (Becton Dickinson, San José, CA, USA) and measurements were stopped when a total of 10 000 events had been accumulated for each sample.

The percentage of sperm with detectable DNA fragmentation, the DNA Fragmentation Index (%DFI), was calculated, using a dedicated software (SCSASoft; SCSA Diagnostics, Brookings, SD, USA) from the DFI frequency histogram obtained from the ratio between the red and

total (red plus green) fluorescence intensity.[22] Flow cytometer set-up and calibration were standardized using a reference semen sample as described by Spano *et al.*[19] Interday SCSA variability, evaluated on the reference sample after 216 FCM sessions by the coefficient of variation (CV) of the %DFI, was 6.0%. In addition, 358 randomly chosen samples (50.6% of the total) were measured twice in independent FCM sessions. Results from the two measurements were highly correlated (DFI, r=0.96).

Finally, the reliability and stability of SCSA measurements have also been challenged by an external quality control study. The quality control study was based on the blind analysis of three aliquots from seven different donors not selected among the participants in this study. The median intersample variability for %DFI, expressed as CV, was 1.5%.[19]

TUNEL assay. Coded frozen semen samples from the three study regions were shipped on dry ice to the FCM facility of Polytechnic University of Marche (Ancona, Italy) for TUNEL analysis.

Briefly, TUNEL assay is a method for detecting DNA fragmentation exploiting terminal deoxynucleotidyl transferase, an enzyme that catalyzes the addition of labelled dUTPs at the DNA break sites.

The pellet obtained previously was fixed in 100 µl of fresh 1% (w/v) paraformaldehyde (PFA) in PBS on a shaker for 1 h at 4°C and then washed with 4 ml washing solution, centrifuged (4°C, 700*g* for 8 min) and the supernatant was discarded. The pellet was then permeabilized with 100 µl of 0.1% (w/v) Na-citrate/Triton-X 100 (permeabilization solution) for 2 min at 4°C; subsequently, 50 µl suspension was drawn for the negative control and the two new aliquots were washed with 0.1% (w/v) PBS/BSA, centrifuged as above and the supernatant discarded. To both tubes, we added 40 µl reaction mix (with and without the deoxynucleotidyl transferase enzyme for the positive and negative samples, respectively) and incubated for 1 h at 37°C in the dark. The pellet obtained after the washing step was fixed with 100 ml of 0.5% (w/v) PFA containing 1 µl ml^{-1} propidium iodide solution in PBS. Samples were stored at 4°C in the dark overnight until the FCM analyses. A minimum of 10 000 sperm were measured by FCM (Epics XL flow cytometer; Beckman Coulter-IL, Fullerton, CA, USA). The intralaboratory CV regarding the TUNEL assay performance was constantly under 5%. Further details are described by Stronati *et al.*[17]

Determination of sperm Fas and Bcl-xL positivity

As for the TUNEL assay, coded frozen semen samples from the three study regions were shipped on dry ice to the FCM facility of Polytechnic University of Marche (Ancona, Italy) for Fas and Bcl-xL analysis.

Sperm cells were fixed with 100 µl 2% (w/v) PFA on a shaker for 20 min on ice for Bcl-xL and 1% solution for 10 min at 4°C for Fas. Subsequently, for the negative control, 50 µl of the suspension was drawn; the cells were washed and centrifuged as above.

To the Bcl-xL sample tube, 100 µl permeabilization solution and 0.5% (w/v) BSA with and without primary monoclonal antibody anti-Bcl-xL (20 µg ml^{-1}) were added for the sample and the negative control, respectively.

To the Fas sample tube, 40 µl anti-Fas primary monoclonal antibody diluted in 0.5% (w/v) PBS/BSA and 40 µl of 0.5% (w/v) PBS/BSA to the negative control tube were added.

After 1-h incubation at 37°C, both the samples and the negative controls were washed and the pellets resuspended in the diluted secondary antibody, for Bcl-xL 50 µl goat anti-mouse IgG-PE conjugated 1 : 100 in PBS+0.3% (w/v) BSA, and for Fas 100 µl goat anti-mouse IgG-FITC conjugated; 1 : 100 in PBS+0.5% (w/v) BSA and incubated for 1 h at 37°C.

After the final washing step, the cells were fixed in 100 µl of 0.5% PFA (w/v). The Bcl-xL samples were stained with 7-aminoactinomicyn D (100 µg ml^{-1} on use) and kept in the dark at 4°C until run on the FCM machine (24 h). The Fas fixed samples were stored in the dark at 4°C (24 h) and 15 min before the FCM analysis, the suspension was stained with 1 µg ml^{-1} propidium iodide.

Two different control ejaculates, stored frozen at −80°C in the laboratory, were thawed and processed according to the Bcl-xL and Fas protocol before starting every FCM run which ensured standardization and stability of the instruments. The intralaboratory CV regarding the apoptotic markers was in the range from 6% for Fas to 9% for Bcl-xL.[17]

All reagents used were molecular biology grade. The primary and secondary antibodies were from Instrumentation Laboratory (Milan, Italy); BSA fraction V, PBS, Na-citrate, Triton X-100, propidium iodide from Sigma Pharmaceuticals (Sigma-Aldrich, Milan, Italy), 7-AAD from Molecular Probes (Invitrogen, Milan, Italy) and secondary antibody blocking peptide from Santa Cruz Biotechnology (CA, USA).

The amount of missing values for Bcl-xL and Fas from Poland (49.7% and 37.6%, respectively) and Ukraine (78.4% and 32.7%, respectively) were high due to accidental loss of samples during shipment and insufficient number of cells for analysis. In Greenland, 27.6% and 3.0% of Bcl-xL and Fas were missing due to few cells for analysis as well.

A minimum of 10 000 sperm were measured by FCM (Epics XL flow cytometer; Beckman Coulter-IL).

Statistical analysis

General linear models (Proc GLM) were used to analyze crude and adjusted associations of demographic, seminal and hormonal characteristics with the two apoptotic markers, Fas and Bcl-xL. Analysis of trends between levels of determinants and apoptotic markers were based on continuous variables.

Observations from the three study regions, Greenland, Poland and Ukraine, were initially analysed separately but are presented in aggregate because correlations were similar across regions as observed by scatter plots. Results for the separate analysis in each study region will not be shown.

Demographical characteristics included study region, age groups ($\leqslant$25, 25–34, $\geqslant$34 years), body mass index ($\leqslant$24.9, 24.9–29.9, $\geqslant$29.9 kg m^{-2}), caffeinated drinks ($\leqslant$5, >5 cups/day), sexual abstinence time ($\leqslant$3, >3 days), spillage during semen collection (yes/no), cotinine in serum ($\leqslant$1.4, 1.4–190, $\geqslant$190 ng ml^{-1}), fever during the last three month (yes/no), genital infections (yes/no) and testicular disorders (yes/no). Seminal characteristics included DFI ($\leqslant$10, 10–20, $\geqslant$20%), TUNEL (0–3, 3–10, >10%), total sperm count ($\leqslant$60, 60–120, $\geqslant 120\times10^6$), sperm concentration ($\leqslant$20, 20–40, $\geqslant 40\times10^6$ ml^{-1}), volume ($\leqslant$2, 2–4, $\geqslant$4 g), immature sperm cells ($\leqslant$1, >1%), sperm cells with normal morphology ($\leqslant$4, 4–14, $\geqslant$14%), head defects ($\leqslant$90, 90–95, $\geqslant$95%), midpiece defects ($\leqslant$10, 10–20, $\geqslant$20%), tail defects ($\leqslant$5, 5–10, $\geqslant$10%), motility ($\leqslant$50, >50%), Fas ($\leqslant$10, 10–50, $\geqslant$50%) and Bcl-xL ($\leqslant$5, 5–40, $\geqslant$40%). Seminal covariates were categorized according to clinical cut-off values or tertiles. All hormonal characteristics were divided into tertiles and included testosterone, estradiol, inhibin-B, FSH, LH and SHBG.

The two outcome variables, Fas and Bcl-xL, were analyzed on a continuous scale. Residuals of Fas and Bcl-xL were checked for normality and were transformed by the natural logarithm.

All crude analyses were adjusted for study region. In the multiple general linear models, we adjusted for study region, age groups, body mass index, caffeinated drinks, cotinine in serum, fever during the last three month, sexual abstinence time, genital infections, testicular disorders and spillage during semen sampling, all divided as described above.

Statistical analysis was performed with SAS software, version 9.1 for Windows (SAS Institute Inc., Cary, NC, USA).

RESULTS

Four hundred and fifty-four men from Greenland, Poland and Ukraine had completed Fas results (3.0%, 37.6% and 32.7% missing, respectively), whereas Bcl-xL observations only were available for 243 men from Greenland and Poland (27.6% and 49.7% missing, respectively).

Men from Poland had approximately a doubling of percentage Fas-positive sperm cells compared with Greenland and Ukraine. Otherwise no demographic characteristics were associated with percentage of Fas-positive sperm cells (**Table 1**). However, several seminal characteristics and reproductive hormones were associated with Fas-positive sperm cells in both bivariate analysis and after adjustment for demographic and clinical characteristics (**Tables 2 and 3**). Sperm concentration, total sperm count ($P=0.01$) and motility ($P=0.04$) were positive correlated with Fas, whereas sperm head defects were negatively correlated with Fas ($P=0.05$), both before and after adjustment for potential confounding effects (**Table 2**). Normal sperm morphology was in the crude analysis significant positively correlated with Fas ($P=0.04$), but not after adjustment ($P=0.07$) (**Table 2**). Results were consistent across study regions except the correlation between sperm count and Fas, which was weaker in Poland and Ukraine (data not shown). Fas positivity was not associated with sperm DNA damage either evaluated by SCSA or the TUNEL assay.

Negative correlations between FSH, testosterone, SHBG and Fas were statistically significant ($P<0.05$) with correlation coefficients near -0.2 (**Table 3**). The three correlations were all consistent within each study region (data not shown). Only FSH was statistically significant when grouped into tertiles ($P=0.021$) (**Table 3**).

Bcl-xL and Fas were positively correlated on a continuous scale ($r=0.2$, $P<0.05$) (data not shown). We found no significant associations between any reproductive hormones, demographical and/or clinical characteristics and the anti-apoptotic marker Bcl-xL (data not shown).

Table 1 Association between Fas and demographic and clinical characteristics

	Fas % positivity					
	n	*Mean*	*Median*	*Range*[a]	P_{crd}	P_{adj}[b]
Site						
Greenland	193	25.0	19.0	91	<0.01	<0.01
Poland	123	48.6	42.3	98		
Ukraine	138	28.2	17.3	98		
Age (year)						
18–25	117	27.0	17.9	95	0.49	0.67
26–34	239	34.3	24.3	98		
35–51	92	35.2	23.6	96		
Body mass index (kg m^{-2})						
18.5–24.9	221	29.1	18.9	98	0.10	0.12
25–29.9	180	34.4	24.6	98		
>30	48	39.6	33.3	95		
Caffeinated drinks (cups/day)						
<5	284	33.2	22.8	98	0.95	0.70
>5	146	30.7	21.2	98		
Alcohol (drinks/week)						
<14	239	37.3	26.6	98	0.11	0.07
>14	32	27.1	17.8	80		
Current smokers						
Yes	265	29.5	19.0	98	0.31	0.24
No	185	36.4	26.8	98		
Cotinine (ng ml^{-1})						
<1.4	185	35.9	24.0	98	0.60	0.73
1.5–190.0	121	32.9	24.9	98		
>190	148	27.6	18.0	98		
Fever						
Yes	45	31.4	26.6	97	0.50	0.30
No	400	32.6	22.2	98		
Genital infections[c]						
Yes	175	25.0	19.0	94	0.05	0.20
No	279	37.0	24.3	98		
Testicular disorders[d]						
Yes	10	28.3	17.6	94	0.55	0.95
No	444	32.5	22.9	98		

[a] The range is the difference between the highest and lowest number of Fas in each category of demographic or clinical characteristics.
[b] Adjusted for site, age groups, body mass index, caffeinated drinks, cotinine in serum, fever the last three month, spillage during semen collection, sexual abstinence time, genital infections and testicular disorders.
[c] Genital infections: epididymitis, gonorrhea, chlamydia or mumps in adulthood.
[d] Testicular disorders: treatment for retracted testis, surgery for varicose veins, torsio testis or testis cancer.

DISCUSSION

This study is to our knowledge the first large study of seminal apoptotic markers in a population of fertile men. Our results show that better semen quality is correlated with a higher percentage of Fas-positive spermatozoa.

Previous studies have investigated Fas expression of ejaculated spermatozoa among infertile men or in men presenting different degrees of alterations in semen parameters compared with fertile controls. In the majority of the studies, Fas was not found on ejaculated sperm cells in normozoospermic men,[23–25] or if present, only in low percentages.[26] Some studies did not even observe Fas on spermatozoa from infertile men[23] or only at low percentages.[25] Studies have found Fas-positive sperm cells in infertile men, and reported inverse relationships between Fas and normal sperm cell morphology, concentration and motility.[14,24,26] These findings indicate that abortive apoptosis may have taken place. Thus, our results are not concordant with previous results. In fertile men from the present study, Fas was present in 96% of the semen samples and correlated with semen parameters, indicating that high Fas levels were positively associated with good semen quality. This could indicate that spermatozoa of fertile men are resistant to Fas-mediated apoptosis and that Fas is inactive or expresses molecules that inhibit the signal induced by Fas or the death program itself.[27,28] In fact, Fas-mediated apoptosis is partially inhibited by overexpression of Bcl-2.[27] However, this does not explain the positive associations observed in the present study. Our observations, however, support a study of three fertile bulls, where high levels of Fas antigen in ejaculated spermatozoa were found. This suggests that Fas antigen in mature, normal ejaculated spermatozoa might evoke a non-apoptotic response against cell death stimuli or to cell-damaging micro-environmental

Table 2 Association between Fas and seminal characteristics

	Fas % positivity							
	n	*Mean*	*Median*	*Range*[a]	P_{crd}	P_{trend}	P_{adj}[b]	r[c]
TUNEL-positive (%)								
0–3	144	28.9	20.5	98	0.15	0.06	0.45	0.08
3–10	136	34.9	24.0	98				
>10	145	35.0	22.9	97				
DFI (%)								
0–10	266	34.2	24.7	98	0.06	0.08	0.11	−0.1
11–20	135	30.6	19.0	96				
>20	52	28.4	16.1	96				
Bcl-xL (%)								
<5	73	30.7	21.1	95	<0.01	0.65	0.01	0.03
6–40	126	34.6	20.9	98				
>40	78	31.6	25.2	98				
Total sperm count ($\times 10^6$)								
<60	35	17.4	7.4	97	<0.01	<0.01	<0.01	0.2**
61–120	89	28.1	18.3	93				
>120	329	34.9	24.9	98				
Sperm concentration ($\times 10^6$ ml^{-1})								
<20	39	13.1	8.3	54	<0.01	<0.01	<0.01	0.2**
20–40	74	26.7	16.8	97				
>40	340	35.6	25.8	98				
Volume (g)								
<2	73	33.9	22.9	98	0.12	0.51	0.34	−0.04
3–4	211	32.8	23.4	98				
>4	170	31.2	19.7	98				
Immature sperm (%)								
<1	251	34.7	23.7	98	0.94		0.81	−0.1
>1	201	29.6	19.5	98				
Normal morphology (%)								
<4	144	27.0	18.4	97	0.04	0.03	0.07	0.2*
4–14	274	34.6	24.0	98				
>14	34	38.5	34.9	98				
Head defects (%)								
<90	160	36.1	27.1	98	0.02	0.02	0.05	−0.1*
91–95	179	32.6	23.4	98				
>95	113	27.0	17.6	97				
Midpiece defects (%)								
<10	31	25.7	12.8	96	0.02		0.02	−0.1
11–20	272	35.3	24.9	98				
>20	149	28.6	18.9	97				
Tail defects (%)								
5	180	30.8	20.2	97	0.42	0.93	0.78	−0.00
6–10	188	33.5	21.8	98				
>10	86	33.0	23.9	98				
Motility (%)								
<50	309	30.2	18.3	98	0.02		0.04	0.1*
>50	145	37.0	31.6	98				
Abstinence time (days)								
<3	143	30.2	19.8	98	0.99		0.66	0.02
>3	276	31.3	22.2	98				
Spillage								
No	412	32.4	22.2	98	0.57		0.43	0.01
Yes	42	32.6	23.3	94				

Abbreviations: DFI, DNA Fragmentation Index; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labelling.
[a] The range is the difference between the highest and lowest number of Fas in each category of seminal characteristics.
[b] Adjusted for site, age groups, body mass index, caffeinated drinks, cotinine in serum, fever the last three month, spillage during semen collection, sexual abstinence time, genital infections and testicular disorders.
[c] Spearmans rank correlation (*$P<0.05$, **$P<0.0001$).

factors like oxidative stress.[29] The same authors also found FasL (the Fas ligand) on ejaculated spermatozoa, indicating co-expression of Fas and FasL in the same cells.[30] They hypothesized that Fas and FasL play different, independent roles in sperm cells. Possible FasL in bull spermatozoa is able to kill activated lymphocytes, and thereby protect the male gamete from damage by the immune system or by the cytotoxic

Table 3 Association between Fas and reproductive hormones

	Fas % positively								
	n	*Mean*	*Median*	*Range*[a]	P_{crd}	P_{trend}	P_{adj}[b]	P_{trend}	r[c]
Testosterone (nmol l^{-1})									
3–13	122	40	34	97	0.19	0.002	0.62	0.10	−0.18*
13–18	124	33	23	98					
>18	111	24	16	94					
Estradiol (pmol l^{-1})									
33–64	123	32	23	98	0.93	0.90	0.87	0.61	0.02
64–81	123	33	22	98					
>81	110	33	23	96					
Inhibin-B (ng l^{-1})									
22–150	114	33	21	98	0.09	0.43	0.051	0.10	0.02
150–203	129	33	23	98					
>203	114	31	23	97					
FSH (IU l^{-1})									
0–3	112	40	35	98	0.03	0.001	0.021	0.002	−0.17*
3–5	135	32	20	97					
>5	110	27	19	96					
LH (IU l^{-1})									
1–3	124	34	22	98	0.67	0.70	0.78	0.93	−0.05
3–5	113	33	24	98					
>5	120	30	20	98					
SHBG (nmol l^{-1})									
6–21	121	38	27	98	0.07	0.004	0.28	0.07	−0.21*
21–31	117	34	24	98					
>31	118	26	18	98					

Abbreviations: FSH, follicle-stimulating hormone; LH, luteinizing hormone; SHBG, sexual hormone-binding globulin.
[a] The range is the difference between the highest and lowest number of Fas in each category of reproductive hormones.
[b] Adjusted for site, age groups, body mass index, caffeinated drinks, cotinine in serum, fever the last three month, spillage during semen collection, sexual abstinence time, genital infections and testicular disorders.
[c] Spearmans rank correlation (*$P<0.05$).

activity of leukocytes in the female genital tract.[30] It would be of great interest to investigate if FasL is present on human ejaculated spermatozoa from fertile men.

In the present study, we did not find associations or determinants of Bcl-xL and demographic, seminal characteristics or reproductive hormones. Likewise, previous studies investigating pro-apoptotic proteins in the Bcl-2 family did not observe associations between Bcl and sperm concentration, morphology or motility.[12] However, Fas and Bcl-xL were positively correlated. Our observations support findings by Sakkas *et al.*[14] with significant positive correlations between Fas and Bcl-x. Bcl-x can be either the long (xL) or the short (xS) form. They investigated which form was expressed in their samples. Bcl-x and Bcl-xL were significantly correlated indicating that Bcl-xL was present. Concomitant high levels of Bcl and Fas might indicate that Bcl is activated in an attempt to turn off apoptosis.

Like in our study, Sakkas *et al.*[14] did not find an association between Fas and TUNEL positivity. Our results also lacked association with the SCSA, the other assay deployed to assess independently sperm chromatin integrity. Furthermore, other studies have not found any relationship between Fas and DNA damage.[24] In a study of 620 Chinese men seeking fertility treatment the authors hypothesized that genetic variants in *Fas*, *FasL* and *Casp8* might contribute to abnormal spermatogenic apoptosis, measured by TUNEL assay, followed by poor semen quality, which could give rise to male infertility.[31] They found that polymorphisms of *Fas*-670GG and *Casp8*-652 6N ins/del were associated with low level of apoptosis in sperm cells and poor semen quality. Germ cells from men carrying both the *Fas*-670G and *Casp8*-652 6N del alleles, i.e., with reduced expression of both Fas and Casp8, displayed lower levels of apoptosis compared to those carrying only one of the alleles.[31] These men are therefore at the lowest risk of sperm apoptosis.[31] A study of *Fas*, *FasL* polymorphisms and azoospermia or severe oligoospermia found that individuals with *FasL*-844TT genotype were more susceptible to azoospermia or severe oligozoospermia than those with *FasL*-844CC genotype.[32] These findings indicate that apoptosis of germ cells may be dependent on genetic diversity. It would therefore be relevant to compare genotypes in fertile and infertile men.

Cross-sectional studies of semen quality suffer often of low participation rates which may bias the internal validity of a study. Subfertile men might be more motivated to participate, but the nature of this cross-sectional design, with data collection through two years, might be predominated by highly fertile couples. However, selection bias among participating and non-participating men is unlikely, since the length of time to pregnancy did not differ among couples where men provided a semen sample in comparison with couples where men declined to deliver semen.[17]

The participation rate of women in the original study varied from 90% in Greenland to 26% in Ukraine. The low participation rate in Kharkiv was a consequence of the recruitment procedure where contact between potential participants and the project team were managed by approximately 30 medical doctors at the three hospitals and eight antenatal clinics. With this large organization, a high level of information and encouragement to participate was not possible. Demographic and reproductive information was obtained from a sample of 605 of those women that declined participation in the study. Only the average age was slightly lower among non-participating women [(22.8 ± 2.4) versus (24.9 ± 2.8) years].[15]

The amount of missing Fas and Bcl-xL from especially Poland and Ukraine were high due to accidental loss of samples during shipment and insufficient numbers of cells for analysis. The loss was random and is thus not expected to bias the results.

The contrasting results from this and previous studies, i.e., high levels of Fas in men with good semen quality and the high percentage of Fas-positive spermatozoa in the ejaculate, might be due to different study populations and/or possible methodological differences in cell fixation, primary antibody clone, fluorescence-labeled secondary antibodies, FCM analysis, software for the off-line analysis to obtain the fraction of positive cells, and the criteria used to define positivity from the FCM data.

This might also indicate that there are differences in the molecular markers of apoptosis between males with normal and abnormal semen parameters and that Fas receptor molecules might be non-functional or non-operative after ejaculation or even protecting the spermatozoa.

CONCLUSION

Ejaculated spermatozoa exhibiting DNA damage do not necessarily show distinct apoptotic markers. Apoptosis and DNA damage in fertile and subfertile men may therefore not be strictly related. These results, carried out in a cohort of spouses of pregnant women, indicate that Fas-positive sperm cells are associated with better semen quality. The results seem different from previous data obtained in subfertile men and warrant further investigation to clarify the biological significance of sperm apoptotic markers.

AUTHOR CONTRIBUTIONS

The study was conceived by GCM, DB, GT and JPB. All authors contributed to design and analyses. Laboratory analyses of apoptotic markers and TUNEL assay were performed by DB and GCM, and MS performed analyses of DNA fragmentation index. GT and AG were responsible for conventional semen analysis and AG was responsible for lab analyses of hormones. IOS, KSH and JPB compiled the data and performed biostatisical analyses. IOS drafted a first version of manuscript and tables and all authors contributed with amendments and revisions.

COMPETING FINANCIAL INTERESTS

None of the authors have competing financial interest in the results of this study.

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