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Cellular maturity and apoptosis in human sperm: creatine kinase, caspase-3 and Bcl-$_{XL}$ levels in mature and diminished maturity sperm

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The relationship between human sperm maturity and apoptosis is of interest because of the persistence of immature sperm in ejaculates in spite of various apoptotic processes during spermatogenesis. We assessed sperm maturity by HspA2 chaperone levels, and plasma membrane maturity by sperm binding to immobilized hyaluronic acid (HA). We also utilized objective morphometry. Sperm were stained with three antibody combinations: active caspase-3/creatine kinase (CK, a marker of cytoplasmic retention), caspase-3/the antiapoptotic Bcl-$_{XL}$, and CK/Bcl-$_{XL}$. In semen, 13% of sperm stained with CK, caspase-3 or Bcl-$_{XL}$, and 28% had stained with two markers. In the mature HA-bound sperm fraction, <4% were single- or double-stained. Regarding sperm regions, CK staining, whether alone or as double staining, occurred in the head and midpiece (15–20%), whereas caspase-3 and Bcl-$_{XL}$ were primarily (>80% of sperm) in the midpiece. Morphometrical attributes of clear, single- and double-stained sperm, in line with their more pronounced maturation arrest, showed an incremental increase in head size (due to cytoplasmic retention) and shorter tail length. We hypothesize that during faulty sperm development, three alternatives may occur: (i) elimination of aberrant germ cells by apoptosis; (ii) in surviving immature cells, caspase-3 is activated, and in response the antiapoptotic Bcl-$_{XL}$, and perhaps HspA2, provide protection; (iii) in a third type of immature sperm, in addition to the CK, caspase-3 and Bcl-$_{XL}$ expression, there are related manifestations of increased head size and shorter tail length. Thus, immature sperm may vary in the type of developmental arrest and in protection mechanisms for apoptosis. These variations are likely to explain the persistence of immature sperm in the ejaculate.

Key words: apoptosis/hyaluronic acid/maturity/morphometry/spermatogenesis/HspA2

Introduction

Spermatogenesis, a complex process of male germ cell development, encompasses spermatogonial proliferation, meiosis and spermiogenesis. The spermiogenetic events that eliminate the surplus cytoplasm, such as development of the acrosome, tail growth, along with cytoplasmic extrusion, result in mature sperm (de Kretser *et al*., 1998; Huszar *et al*., 2000). Developmental defects may occur in both the cytoplasmic or nuclear compartments, which can result in the production of immature sperm.

Creatine kinase (creatine phosphokinase, CK) in human sperm is a marker of cytoplasmic retention and, thus, diminished sperm maturity (Huszar and Vigue, 1990, 1993). In unison with cytoplasmic extrusion, there is also a remodelling of the sperm plasma membrane (Huszar *et al*., 1997). Functional evidence for the remodelling process originates in studies of CK-immunostained sperm–hemizona (halved unfertilized human oocytes) complexes. Immature sperm with cytoplasmic retention were not able to bind to the zona pellucida, suggesting that the formation of the zona-binding site(s) is part of the membrane remodelling process (Huszar *et al*., 1994, 1997). Subsequently, we have also demonstrated that synthesis of the binding sites for hyaluronic acid (HA), a component of the female reproductive tract, is also regulated by the plasma membrane-remodelling events (Huszar *et al*., 1990; Sbracia *et al*., 1997). Mature sperm that bind to HA do not show cytoplasmic retention, are of normal morphology, have high DNA integrity and a low frequency of chromosomal aneuploidies (Huszar *et al*., 1997, 1998, 2003; Kovanci *et al*., 2001).

In addition, independent observations in our laboratory have indicated that sperm shape is very closely related to maturity. Sperm of diminished maturity have larger, rounder and amorphous heads due to the cytoplasmic retention (Huszar and Vigue, 1993). As sprouting of the tail is also a spermiogenetic event, immature sperm also show shorter tail lengths. In two studies, the ratio of tail length:long head axis was shown to be a very sensitive marker of sperm maturity (Gergely *et al*., 1999; Celik-Ozenci *et al*., 2003). Furthermore, mature sperm that are able to bind to HA compared to the non-binding sperm significantly differ in all tail, head, tail:head long axis ratio parameters (Celik-Ozenci *et al*., 2002). This relationship is also demonstrated in the present work.

Another important marker of sperm maturation is the testis-specific Hsp70-2, a member of the highly conserved Hsp70 family of chaperone proteins. In mice, the targeted disruption of the HSP70-2 gene caused the fragmentation of the synaptonemal complexes (structures formed between homologous chromosomes during the meiotic process), and a failure of desynapsis of the paired chromosomes (Dix *et al*., 1996). A second wave of 70 kDa chaperone expression (hsc70t in mice) was also demonstrated during terminal spermiogenesis (Dix *et al*., 1996; Eddy, 1999). In addition to the meiotic support, the chaperone proteins are also responsible for the transport and folding of proteins, such as DNA repair enzymes, or the proteins for sperm membrane remodelling. HSP70-2 also exhibits an anti-apoptotic function (Buzzard *et al*., 1998; Garrido *et al*., 2001; Parcellier *et al*., 2003). Indeed, in Hsp70-2 knockout mice, in addition to arrested meiosis, there is increased germ cell apoptosis with the characteristic apoptotic DNA degradation pattern (Dix *et al*., 1996).

The role of the Hsp70 chaperone proteins in regulating the apoptotic process is well studied in somatic cells. In cell cultures, Hsp70 antisense oligomer caused an inhibition of Hsp70-1 expression and promoted apoptosis, whereas heat or other stress induced Hsp70 synthesis with a reduction of apoptosis (Wei *et al*., 1994, 1995). The mechanism of Hsp70 action in apoptosis is related to the apoptotic enzymes caspase-3 and -9. Activated caspase-9 is associated with cytochrome c that is released from the mitochondria. Both the apoptosis-triggering effect of cytochrome c upstream and the recruitment of caspase-9 to the apoptosis activating complex downstream, are inhibited by the C-terminal region of Hsp70. Thus, the Hsp70 chaperones, such as the homologous HSP70-2 as well as the human HspA2, are likely inhibitors of the apoptotic process (Beumer *et al*., 2000; Li *et al*., 2000).

The identity of HspA2 and the two-wave expression pattern of the testis-specific chaperone proteins in spermatogenesis and spermiogenesis has been established in men (Huszar *et al*., 2000). HspA2 first appears in the primary and secondary spermatocytes as a component of the synaptonemal complex. The second wave of HspA2 (perhaps hsc70t as in the mouse) expression occurs in elongating spermatids, simultaneously with cytoplasmic extrusion and plasma membrane remodelling. In ejaculated human sperm with low levels of HspA2, there are several other markers that reflect diminished maturity. These include cytoplasmic retention and consequential abnormal sperm head shape, diminished binding to the zona pellucida and HA, increased rate of chromosomal aneuploidies caused by meiotic defects, increased levels of DNA fragmentation, retarded histone-protamine replacement, and shape attributes detected by objective morphometry that are characteristic for immature sperm (Celik-Ozenci *et al*., 2003; Huszar *et al*., 2003; Kovanci *et al*., 2001; Óvári *et al*., 2003).

Several studies have focused on various aspects of sperm immaturity, DNA damage and apoptosis in sperm (Aitken *et al*., 1994; Huszar and Vigue 1994; Manicardi *et al*., 1995, 1998; Huszar *et al*., 1998; Sakkas *et al*., 1999a,b; Gandini *et al*., 2000; Irvine *et al*., 2000; Aitken and Krausz, 2001; Ricci *et al*., 2002; Shen *et al*., 2002). In line with the general notion that the proportion of immature sperm is higher in samples with lower sperm concentration, Sakkas *et al*. (2002) have demonstrated an inverse correlation between sperm concentrations and a number of apoptotic marker proteins; however, a potential link between the morphological attributes of such sperm and their apoptotic profile is yet unexplored.

In considering the relationship between apoptosis and diminished sperm maturity, we hypothesized that there may be a relationship between cytoplasmic retention, larger heads and shorter tails and the presence of various apoptotic proteins in the same sperm. In the present study we have therefore investigated the relationship between diminished sperm maturity and apoptosis. We have applied double immunostaining for combinations of CK/caspase-3, Bcl-$_{XL}$/caspase-3 and CK/Bcl-$_{XL}$ in mature and diminished maturity sperm fractions characterized by their level of CK and HspA2 content. Subsequently, we determined the morphometrical attributes of sperm with various staining patterns originating in semen and in the HA-bound mature sperm fractions.

Materials and methods

Patient population

The study samples originated from the leftover portion of semen submitted for semen analysis at the Sperm Physiology Laboratory, Department of Obstetrics and Gynecology, Yale University of Medicine. Samples were collected by masturbation after 2 days of abstinence, and were allowed to liquefy for 30–60 min. All studies were approved by the Yale School of Medicine Human Investigation Committee.

After determination of sperm concentration and motility according to World Health Organization (1999) criteria, the semen was diluted with 3 volumes of human tubal fluid medium (HTF; Irvine Scientific, USA) containing 0.5% bovine serum albumin (BSA). The diluted semen was centrifuged at 500 *g* for 15 min at room temperature. The sperm pellet was resuspended in HTF, and aliquots were taken for CK activity and HspA2 ratio determinations. Sperm smears were prepared for various studies, including CK immunocytochemistry for cytoplasmic retention and determination of Bcl-$_{XL}$ and active caspase-3 expression levels by immunocytochemistry. For preparation of the HA-bound mature sperm fractions, 7 µl aliquots were applied to HA-coated glass slides (Biocoat, Inc., USA). After incubation for 10 min in a humidity chamber, the slides were washed gently in order to remove the unbound sperm (Huszar *et al*., 2003). The smears of unselected and HA-selected sperm fractions were air-dried and fixed with formaldehyde for the CK, caspase-3 and Bcl-$_{XL}$ immunocytochemical assessments.

In the initial and respective HA-bound sperm fractions, we evaluated 200 cells for staining intensity as light mature (L), slightly dark (S) or dark immature (D), whether the slides were processed for CK retention or Bcl-$_{XL}$ immunocytochemistry.

CK activity and HspA2 ratio measurements

These assays were carried out by standard procedures as described previously (Huszar and Vigue, 1990; Huszar *et al*., 1992). Aliquots of semen were washed with 10–15 vols of 4°C 0.15 mol/l NaCl and 30 mmol/l imidazole (buffer, pH 7.0) at 5000 *g* in order to remove seminal fluid. The sperm pellets were disrupted by vortexing in 0.1% Triton, 30 mmol/l imidazole (pH 7.0), 10% glycerol, and 5 mmol/l dithiothreitol. The homogenate was clarified by centrifugation at 5000 *g*, and aliquots of the extract were subjected to CK activity determinations by a spectrophotometric CK kit (Sigma, USA).

The sperm CK-B isoform and the HspA2 were separated by electrophoresis on precast Agarose gels (Helena Laboratories, USA). The separated proteins were detected by overlaying the gel with a fluorescent ATP substrate. The fluorescent bands corresponding to CK-B and HspA2 were quantified under long-wave UV light with a scanning fluorometer. The HspA2 ratio is expressed as % (HspA2/HspA2 + CK-B).

Immunostaining of sperm for CK, Bcl-x$_L$ and caspase-3

The CK immunocytochemistry procedures have been described previously (Huszar and Vigue, 1993; Huszar *et al*., 1994). Both the initial semen and the HA-selected sperm fractions were fixed with 3.7% formaldehyde in phosphate buffer/sucrose (PB-suc) for 20 min at room temperature. After removal of the formalin, the slides were allowed to air dry. After three washing steps with PB-suc, the sperm were exposed to a 3% BSA (blocking solution) in PB-suc at room temperature. After further washing with PB-suc, the slides were covered with 1:1000 dilution of either of polyclonal anti-CK-B antiserum (Chemicon Co., USA), 1:1000 dilution of active caspase-3 (PharMingen, USA), or 1:100 dilution of monoclonal anti-Bcl-$_{XL}$ antibody (Transduction Laboratories, USA) and incubated overnight at 4°C. After the washing steps, single staining of CK, Bcl-$_{XL}$ and caspase-3 was carried out with second antibodies and the slides were visualized with light microscopy. The specificity of the staining was established by using preimmune serum in place of the first antibody, or by applying the second antibody only.

In order to visualize the simultaneous presence of caspase-3 activity and CK retention or Bcl-$_{XL}$ levels in semen sperm or HA-bound sperm fractions, double labelling was performed with antisera in the same dilution as for single staining. In order to visualize the CK/*Caspase-3*, *Caspase-3*/Bcl-x$_L$ and CK-Bcl-$_{XL}$ double markers, caspase-3 immunoreaction was detected by using a 1/400 dilution of alexa flour-350 goat anti-rabbit secondary antibody (Molecular Probes, USA), CK immunoreaction was detected by using a biotinylated anti-goat second antibody (Vector Laboratories, USA) at a 1:1000 dilution and a

1:200 dilution of F/TC (fluorescein isothiocyanate)-labelled avidin (Vector Laboratories) and Bcl-x_{XL} immunoreaction was detected by using a 1:200 dilution of PE (phycoerythrin)-labelled goat anti-mouse secondary antibody (Molecular Probes, USA).

Objective morphometry measurements by Metamorph

This computer-based program was developed by Universal Imaging Co. (USA). The details of the methods, along with the validation information, are described in Celik-Ozenci *et al*. (2003).

Briefly, calibration of the system was performed by viewing an objective micrometer scale (OB-M 1/100) at ×100 magnification, and digitizing the image with the Metamorph™ program. The automated conversion of pixels to µm was 0.13 µm/pixel.

After capturing and digitizing the images, Metamorph™ overlay tools were used to delineate the head versus tail regions of individual sperm in order to measure head and tail parameters separately. In the assessment of head parameters, Metamorph™ recognizes the following elements: area (area of entire head); perimeter (distance around edge of head, measuring from midpoints of each pixel that defines its border); long head axis (length of longest diameter); short head axis (width measured perpendicular to the longest diameter); shape factor (a value from 0 to 1 representing how closely the object represents a circle, with 1 being a perfect circle). For the sperm tail measurements, Metamorph™ distinguishes the fibre length (the length of an object, assuming that it is a fibre). In addition, in our laboratory, we have developed a parameter which is not standard to the Metamorph™ program, but which reflects well sperm cellular maturity (Gergely *et al*., 1999): tail length/long head axis. These additional parameters were calculated using Microsoft Excel.

Statistical analysis

In order to compare the various sperm attributes—sperm concentrations and other semen parameters, CK-activity, HspA2 ratios, the staining intensity of the CK and Bcl_{XL} biochemical markers (darkness factor as described in Results), and the morphometry measures—we used the *t*-test analysis and non-parametric comparisons, with the computer-based SigmaStat program (Version 2.0; Jandel Scientific Corp., USA). In testing the various correlations, we have used Pearson correlation analysis utilizing the SigmaStat program. $P < 0.05$ was accepted as significant.

Results

Cytoplasmic retention and Bcl-x_L expression in sperm of various maturity

In focusing upon the relationship between the expression levels of Bcl-$_{XL}$ and of other biochemical markers in human sperm, we studied 30 men, sperm concentration 34.8 ± 4.1×10^6 (min–max: 10–106×10^6), motility 53.3 ± 2.5% (min–max: 27–72%, all data mean ± SEM). Based on the sperm HspA2 ratios, we divided the men into three maturity groups: low (*n* = 10), intermediate (*n* = 10) and high (*n* = 10). The sperm maturity markers of HspA2 ratio and CK activity were significantly different in the three groups, whereas sperm concentrations, motility and total motile sperm concentration were similar (Table I). These data are in agreement with our consistent findings: sperm maturity is largely independent of the sperm concentrations (Huszar and Vigue, 1990, 1993; Huszar *et al*., 1994).

From each sample, we prepared sperm smears and the HA-bound mature sperm fractions. Further, we subjected the slides to immunostaining for CK or Bcl-x_L, and evaluated the proportion of sperm according to their staining intensity. In each sample, we assessed 200 sperm (800 sperm/man, 24 000 sperm in 30 men). The staining intensity of sperm was quantified by the 'darkness factor' using the following scale: unstained clear sperm = 0, partially or slightly stained sperm = 1.5, and darkly stained sperm = 3. For instance, considering 200 sperm: 160 clear, 36 slightly stained, and 4 dark, would yield a darkness factor of 66 (36×1.5 + 4×3). The values reported in Table II are based on the cumulative staining intensity of 200 cells in each sample. The clear, slightly or darkly stained sperm are easily distinguishable when all three types are observed on the same slide (Figure 1).

Table I. The mean HspA2 levels and semen parameters in samples with low, intermediate and high HspA2 ratios

	Low HspA2 (*n* = 10)	Intermediate HspA2 (*n* = 10)	High HspA2 (*n* = 10)
HspA2 (%)	**17.8 ± 1.5**	**31.8 ± 1.6**	**60.5 ± 2.6**
CK activity (IU/10^8)	3.2 ± 0.8	2.3 ± 0.9	0.7 ± 0.2[a]
Sperm concentration (10^6/ml)	27.3 ± 5.9	33.6 ± 3.6	43.6 ± 10.3
Total motile sperm (10^6/ml)	13.6 ± 4.1	19.1 ± 2.5	25.8 ± 6.6
Motility (%)	47.4 ± 4.7	56.8 ± 3.7	56.8 ± 3.7

Values are mean ± SEM.
Values in bold: $P < 0.001$ in all comparisons.
[a]$P < 0.05$ high HspA2 versus intermediate HspA2 and low HspA2 (*t*-test).

Table II. The creatine kinase (CK) and Bcl-x_L darkness factor in the semen and HA-selected sperm fractions

	Low HspA2 (*n* = 10)	Intermediate HspA2 (*n* = 10)	High HspA2 (*n* = 10)
Semen			
CK	**61.3 ± 5**[a]	38.5 ± 3.5[a]	38.2 ± 5[a]
Bcl-x_L	**57.5 ± 5**[b]	33.2 ± 4.2[b]	31.9 ± 5.1[b]
HA-bound			
CK	**25.8 ± 4.1**[a]	20.2 ± 3.3[a]	18.5 ± 3.4[a]
Bcl-x_L	**28.0 ± 5**[b]	17.8 ± 2.3[b]	17.0 ± 3.5[b]

Values are mean ± SEM.
Values in bold: $P < 0.05$, low HspA2 versus intermediate HspA2 and high HspA2 ratios (horizontal comparisons).
[a] and [b]$P < 0.001$ in the respective CK and Bcl-$_{XL}$ values of the semen and HA-bound sperm fractions (vertical comparisons), *t*-test.

The study of the sperm populations arising from semen and of the mature sperm selected by HA binding has further supported the relationship between sperm maturity, the expression levels of CK content (cytoplasmic retention) and Bcl-$_{XL}$ expression levels (Table II). Regarding the three HspA2 maturity groups, both the CK and Bcl-$_{XL}$ darkness factors reflected the maturity-related differences, as they were significantly higher in the low versus the less mature intermediate and high staining intensity groups (Table II, horizontal comparisons). The staining intensity in the vertical comparisons between the sample pairs is substantially lower in the HA-bound versus the semen sperm fraction ($P < 0.001$, Table II).

These staining differences between the low, intermediate and high maturity groups arise because in the semen fractions, there is a mixture of sperm with various degrees of maturity. However, in the respective HA-bound sperm fractions, the staining pattern is lighter by virtue of the plasma membrane maturity and higher HA receptor density, which in turn lead to a higher uniformity of the HA-bound sperm (Huszar *et al*., 1994, 1997, 2003).

The common maturity-related origin among CK activity (cytoplasmic retention), HspA2 ratio and Bcl-x_L expression levels were also reflected by the significant correlations between these markers: in semen (CK versus Bcl-$_{XL}$ $r = 0.71$, $P < 0.001$; HspA2 versus Bcl-$_{XL}$ $r = –0.52$, $P = 0.003$; CK versus HspA2 ratio $r = –0.55$, $P = 0.001$). In the HA-bound sperm fraction: CK versus Bcl-$_{XL}$ $r = 0.75$, $P < 0.001$.

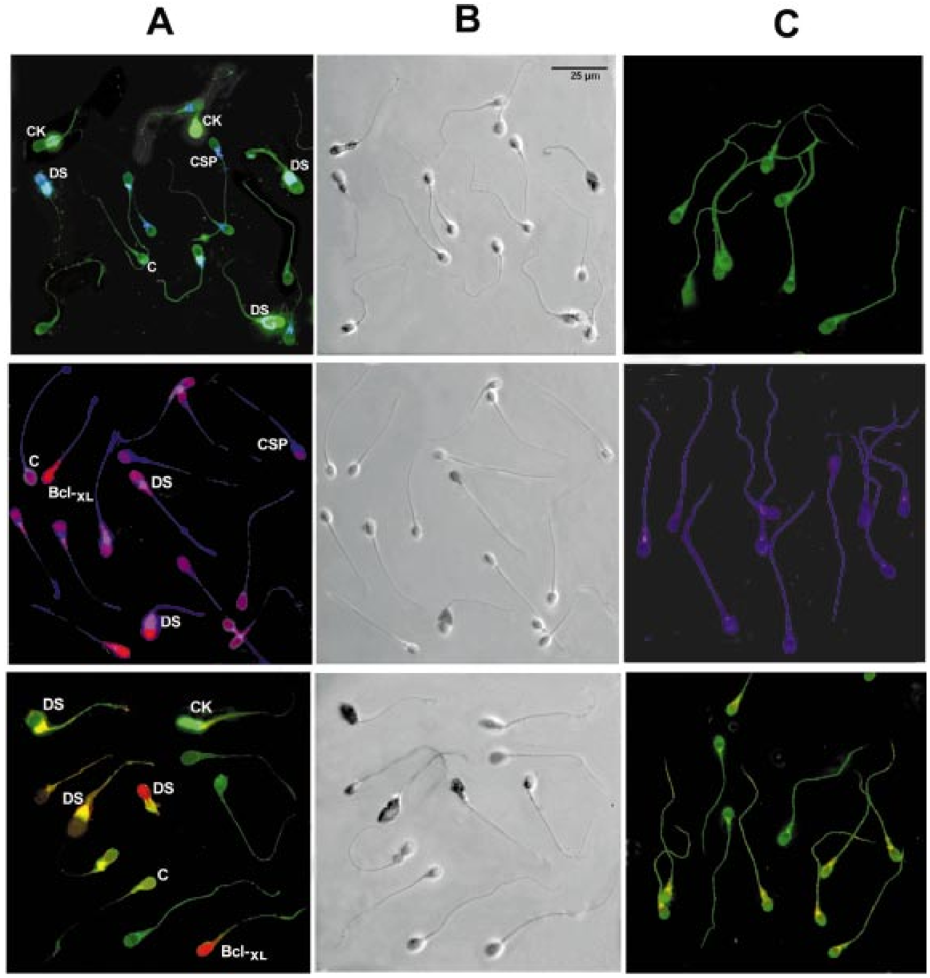

Figure 1. Sperm single- or double-stained for creatine kinase (CK), Bcl-x_L and caspase-3. The fluorescent colour labels are the following: upper row: CK/caspase-3 (green–blue); middle row: caspase-3/Bcl-x_L (blue–red); and lower row: CK/Bcl-x_L (green–red)]. Column A represents sperm originating in semen samples; column B demonstrates the same sperm cells under phase contrast microscopy; and column C represents the respective HA-bound sperm fractions. C = clear; CK = CK positive; CSP = caspase-3 positive; Bcl-$_{XL}$ = Bcl-$_{XL}$ positive; DS = double-stained sperm.

Sperm maturity and levels of active caspase-3 and Bcl-x_L expression

In the next set of experiments, we examined the hypothesis that sperm with diminished maturity may arise either from apoptosis as indicated by the presence of Bcl-$_{XL}$ and active caspase-3, or from a sperm population with a more retarded development exhibiting cytoplasmic retention. We have double-stained semen sperm and their respective HA-bound fractions with the three immuno-marker combinations of *CK/active caspase-3*, *active caspase-3*/Bcl-$_{XL}$ and *CK*/Bcl-$_{XL}$ (Figure 1 upper, middle and lower rows). In the semen fraction (Figure 1A), one can identify sperm with three distinct patterns: clear sperm, single marker-stained sperm with CK, caspase-3 or Bcl-$_{XL}$, and double-stained sperm with the various marker combinations. In the HA-bound mature sperm (Figure 1C), almost all sperm are the clear type.

In order to better understand the respective association between the immunostaining patterns, the HA-binding characteristics and the morphometrical attributes of sperm, we considered three aspects: (i) the types of staining patterns and the proportion of sperm exhibiting particular staining patterns; (ii) the head and midpiece distribution of CK, caspase-3 [with our antibody we are detecting active caspase); (iii) the morphometrical attributes of sperm with various staining patterns.

Staining patterns with CK and active caspase-3 (Figure 1, upper panel)

In the semen fractions we have identified clear sperm, CK-only-stained sperm, caspase-3-only-stained sperm, and sperm that were stained with both CK and caspase-3. In the HA-bound sperm fraction there were a few lightly stained sperm, but no sperm with substantial cytoplasmic retention or caspase activity. This finding is in line with our hypothesis; sperm that stained either for CK or caspase-3 only likely represent sperm subpopulations that are immature due to an early and compensated apoptotic process during spermatogenesis. Conversely, sperm that are double-stained with both CK (cytoplasmic retention) and caspase-3 most likely arise from a combination of arrested development and apoptosis. Finally, it is of note that sperm, whether with the single- or double-staining patterns, are immature as they have not completed membrane remodelling thus are deficient in HA binding.

Sperm double-stained for Bcl-$_{XL}$ and caspase-3, or for CK and Bcl-$_{XL}$ (Figure 1, middle and lower rows), similar to the CK and

Table III. Morphometrical attributes of sperm fractions with various staining patterns (n = 1200 sperm evaluated mean ± SEM)

	Tail length (μm)	Head area (μm²)	Perimeter (μm)	Long axis (μm)	Short axis (μm)	Shape factor	Tail:long axis ratio	*n*
HA-bound	**62.1 ± 0.2**	**20.6 ± 0.2**	**17.9 ± 0.1**	**6.5 ± 0.03**	**4.5 ± 0.02**	0.82 ± 0.0	**9.7 ± 0.0**	450
Clear	56.3 ± 0.4	21.8 ± 0.3	18.5 ± 0.1	6.6 ± 0.05	4.7 ± 0.04	0.81 ± 0.0	8.6 ± 0.1	180
Caspase-3 only	**46.5 ± 0.5**	**24.0 ± 0.4**	**20.1 ± 0.3**	**7.2 ± 0.08**	4.8 ± 0.05[a]	**0.76 ± 0.01**	**6.6 ± 0.1**	190
CK only	**45.5 ± 0.5**	**26.2 ± 0.5**	**21.1 ± 0.3**	**7.7 ± 0.1**	4.9 ± 0.08[a]	**0.74 ± 0.0**	**6.4 ± 0.1**	140
Bcl-$_{XL}$ only	**45.6 ± 0.3**	21.7 ± 0.6	18.8 ± 0.3	6.8 ± 0.1	4.6 ± 0.9	0.77 ± 0.0[a]	**6.9 ± 0.1**	100
Double-stained sperm	**43.6 ± 0.6**	**28.1 ± 0.7**	**22.2 ± 0.4**	**8.0 ± 0.1**	**5.1 ± 0.09**	**0.72 ± 0.01**	**5.6 ± 0.1**	140

The attributes in each column are compared to respective 'clear' type sperm (underlined).
Values in bold: $P < 0.001$; [a]$P < 0.05$ compared to 'clear' (paired *t*-test).

caspase-3 staining, showed the four types of pattern, with sperm heads of clear, caspase-3-only, CK-only, Bcl-$_{XL}$-only, or the Bcl-$_{XL}$-caspase-3 and CK-Bcl-x_L double-staining.

Proportions of sperm that showed the various staining patterns

With respect to the proportion of various staining patterns, we have found a remarkable consistency with the three pairs of immunomarker combinations. In the semen fraction, whether with the CK/caspase-3, caspase-3/Bcl-$_{XL}$ or CK/Bcl-$_{XL}$ combinations (Figure 1, upper, middle and lower rows), the mean proportions of clear and double-stained sperm were 45.1 ± 1.0 and 27.7 ± 0.9% (half of the stained sperm), whereas the single-stained sperm with the various probe combinations in the upper, middle and lower rows represented 20.5 ± 1.5, 14.1 ± 2.1 and 6.5 ± 0.5% respectively.

In each study, 300–500 sperm were evaluated in five randomly chosen men. It is of interest that at least two-thirds of semen sperm that stained with caspase-3 were also double-stained with Bcl-$_{XL}$. In the respective HA-bound mature sperm fractions, >90% of the sperm were the clear type. The other four sperm staining patterns, caspase-3-only, CK-only, Bcl-$_{XL}$-only and double-stained sperm, occurred only at a mean <4% incidence.

Regarding the staining in the sperm head or midpiece or in both regions with the various immunomarkers, the CK staining, representing cytoplasmic retention, was localized in the head (25%), in the midpiece (>60%) and in the head + midpiece (18–20% of sperm, Figure 2). This is in agreement with our previous data (Huszar and Vigue, 1993; Gergely *et al*., 1999). Caspase-only staining in the midpiece has occurred with a >92% incidence, whereas the Bcl-$_{XL}$ only pattern in the midpiece was detected in 75–80% of the sperm. Others also noted the prevalence of caspase-3 midpiece staining (Weng *et al*., 2002).

Morphometrical attributes

Regarding sperm dimensions, there were distinct maturity-related differences between the clear and the caspase-3-only, CK-only, Bcl-$_{XL}$-only and double-stained sperm populations. These morphometrical attributes of the sperm populations have provided independent supporting evidence for our hypothesis regarding the different mechanism of how sperm that are single- or double-stained evolve (1200 sperm originating in five men, Table III). The clear sperm had significantly longer tails, smaller and better-shaped heads, as well as higher tail length:long head axis ratios. The sperm with the three types of single staining patterns had tail and head morphometrical attributes that were consistent with diminished maturity sperm compared to both classes of clear mature sperm. Furthermore, the double-stained sperm had significantly shorter tail length and larger head area, circumference and long axis dimensions ($P < 0.001$), indicating a distinct difference between the single-stained and even less mature double-stained sperm population. The sperm populations double-stained with CK/caspase-3, caspase-3/Bcl-$_{XL}$ or with CK/Bcl-$_{XL}$ firmly support the morphometrical and maturity-related differences between the single-stained versus double-stained sperm.

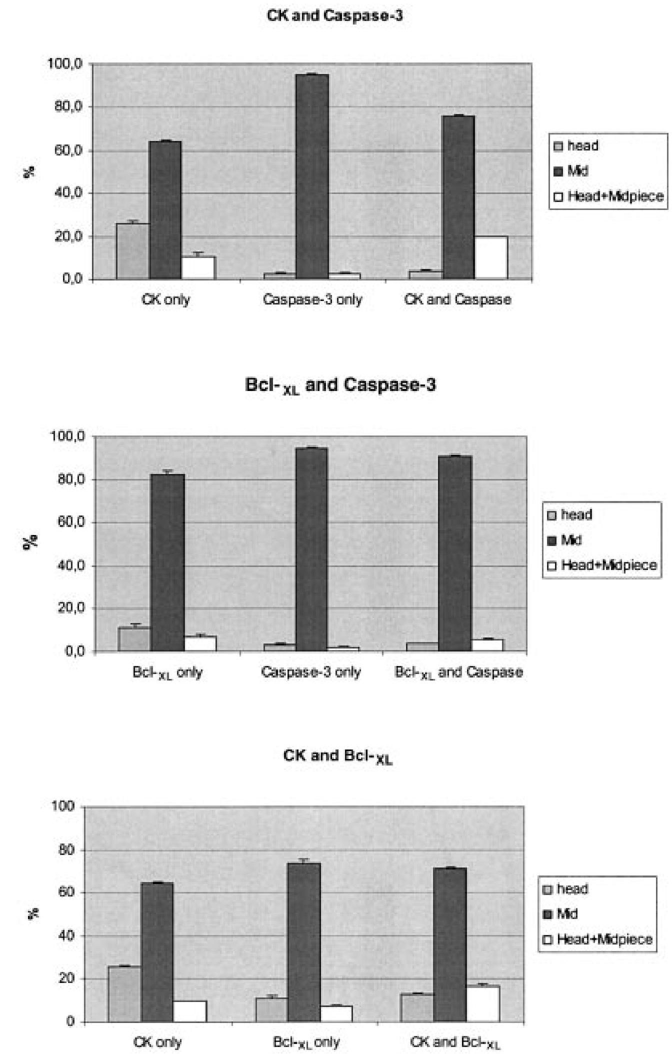

Figure 2. The distribution of head and midpiece staining patterns in sperm exhibiting single or double staining with creatine kinase (CK), Bcl-$_{XL}$ and caspase-3 antisera.

It is of further interest that the HA-bound clear sperm had longer tails, smaller heads and a smaller SEM in all parameters compared to the clear sperm of semen (Table III). These differences are due to the heterogeneity of clear sperm in semen compared to the uniformity of HA-bound sperm, which is the most mature sperm population selected by the highest density of HA receptor.

The maturation-related decline of tail length and increase in head parameters of area, perimeter and long axis in the five types of sperm are most impressive (Table III). Indeed, the tail of the clear semen sperm is >25% longer and the HA-bound clear sperm are 31% longer than that of the mean of the three classes of stained immature sperm. The head area, perimeter and long head axis is ~15% larger in the stained versus the clear sperm fractions. The maturity-related dimensional differences are best demonstrated by the decline of tail length:long head axis ratios in the HA-bound clear, clear, single-stained or double-stained sperm populations (ratios. 9.1 ± 0.0, 8.6 ± 0.1, 6.6 ± 0.1, 5.6 ± 0.1 respectively, independently from the probe combinations used). The ratio of the double-stained sperm is 35% shorter than that of the clear semen sperm, and 44% shorter compared to populations of HA-bound and clear sperm.

The dimensions of the double-stained sperm whether by the CK/caspase-3, Bcl-$_{XL}$/caspase-3 and CK/Bcl-$_{XL}$ combinations were comparable both in the head and in the tail length:long head axis ratio dimensions. This indicates that these double-stained sperm by the various markers are actually 'triple-stained', i.e. the combination of all these markers, CK, caspase-3 and Bcl-x_L staining. This idea is further supported by the dimensions of the CK/Bcl-$_{XL}$-stained sperm compared to the mean of the CK/caspase-3 and caspase-3/Bcl-$_{XL}$ sperm (in parentheses): tail length: 44.9 μm (44.1); head area: 26.2 μm^2 (27.5); perimeter: 20.0 μm (21.1); long axis 7.3 μm (7.9); short axis 5.0 μm (5.1); shape factor 0.78 (0.72); and tail length/head axis ratio: 6.2 (5.8).

Discussion

During spermatogenesis and spermiogenesis, apoptosis, the controlled degradation of sperm DNA, has been suggested to play a key role in adjusting the appropriate number of proliferating germ cells associated with the surrounding Sertoli cells (Blanco-Rodriguez, 1998; Li *et al*., 2000; Print and Loveland, 2000; Kaufmann *et al*., 2001). The regulation of apoptosis is based on the intracellular dominance of various proteins that induce or inhibit the apoptotic process, such as BAX, Bcl-$_{XL}$, caspase-3 and several key enzymes. Bcl-$_{XL}$ has been previously observed in ejaculated sperm (Sakkas *et al*., 2002). In samples with low sperm concentrations that also have higher incidence of immature sperm, a proportionally higher Bcl-$_{XL}$ expression occurs (Huszar *et al*., 1988; Huszar and Vigue, 1990; Sakkas *et al*., 2002). In the current study, we have found that immature sperm show a proportionally higher level of Bcl-$_{XL}$ and caspase-3 expression. Although the current and previous Bcl-$_{XL}$ data suggest that this anti-apoptotic protein is an important factor in the survival of immature germ cells, and this finding is also supported by the regulation of mouse germ cell survival, it is clear that the balance of both pro- and anti-apoptotic members of the Bcl-2 family are involved in the fate of immature sperm that would otherwise be eliminated by apoptosis (Rucker *et al*., 2000; Sakkas *et al*., 2002).

Our laboratory has focused upon the objective biochemical markers, such as CK-activity and HspA2 ratios, for the evaluation of sperm maturity, function and male fertility (Huszar and Vigue, 1993; Huszar *et al*., 1994, 2000; Ergur *et al*., 2002). It has been shown that diminished expression of the HspA2 chaperone protein is associated with cytoplasmic retention and consequential abnormal morphology, along with changes detectable with objective morphometry, such as the increase in head size or shorter tail length (Gergely *et al*., 1999). The correlation between sperm CK activity and HspA2 ratios was consistently close in several studies ($r \approx -0.7$, $P < 0.001$; Huszar *et al*., 1992; Lalwani *et al*., 1996; Ergur *et al*., 2002). The HspA2 family of chaperone proteins facilitates the intracellular transport of proteins in the elongating spermatids, the repair of DNA strand breaks, and likely supports plasma membrane remodelling and the collection and externalization of surplus cytoplasm (Dix *et al*., 1996; Eddy, 1999; Huszar *et al*., 2000). Diminished maturity sperm with low HspA2 levels have extensive DNA fragmentation, increased rates of aneuploidy and diminished fertility (Huszar *et al*., 1994; Kovanci *et al*., 2001). Mature sperm that are able to bind HA show none of the markers of diminished maturity (Huszar *et al*., 2003). In two blinded studies, we have demonstrated that low levels of sperm HspA2 predicts the failure of pregnancies in couples treated with IVF (Huszar *et al*., 1992; Ergur *et al*., 2002).

The present work has furthered our knowledge on the relationship between sperm maturity and the expression levels of CK, caspase-3 and Bcl-$_{XL}$. We were interested in the mechanism which would allow the conservation of grossly immature sperm in the ejaculate. These sperm cells would be expected to be eliminated in the adluminal area or the epididymis by the ongoing apoptotic process. Focusing on maturity, we divided the 30 men into three groups based on their high, intermediate and low HspA2 levels. The data of Table II clearly indicate that there was a relationship between the HspA2 ratios and the biochemical markers in the three maturity groups and also in semen versus the HA-bound sperm fractions. Another relevant aspect, which highlights the factor of maturity, is the correlations between the darkness factor and HspA2 ratios on one hand and of the various apoptotic markers on the other hand. Furthermore, the most mature sperm fractions bound to HA, which have completed membrane remodelling and have a higher density of HA receptors, showed a very low expression of CK, caspase-3 and Bcl-$_{XL}$ (Figure 1C; Huszar *et al*., 1997, 2003).

Regarding the staining patterns, we have observed sperm that were clear and sperm stained exclusively with CK, caspase-3 or Bcl-$_{XL}$, or were double-stained with the CK/caspase-3, caspase-3/Bcl-$_{XL}$ or CK/Bcl-x_L combinations (Figure 1). It is of interest that the proportions of sperm that were clear, or stained with a single or two markers, were consistent with the various probe combinations. Also, >90% of the HA-bound mature sperm were the clear type (Table III).

The regional distribution of the markers in the various sperm types was also different. The midpiece-stained sperm with caspase-3 occurs with a >90% incidence, whereas the Bcl-$_{XL}$ in the sperm midpiece pattern occurs >75% of the time. However, CK staining, representing cytoplasmic retention, is present in the head only or in head and midpiece in ~60% of the CK-containing sperm (Figure 2). These variations in patterns also indicate that the origin of defect that leads to caspase-3/Bcl-$_{XL}$ or CK expression is different. The dimensions of sperm with single- or double-stained patterns were different from that of the clear sperm, whether one considers the 28% decline in tail length or the 15% increase in the head parameters of area, perimeter and long axis (Table III). Thus, if sperm were stained with single or double markers, they were of lesser maturity compared to the clear sperm populations, as is further indicated by the substantial decline in tail length:long head axis ratios in sperm with arrested maturation.

The uniformity in size of the clear, single- and double-stained sperm by the various probe combinations suggests that the double-stained sperm actually contain all three markers, or 'triple-stained', as they are the very same immature sperm highlighted independently by the three sets of biochemical markers. These staining patterns, regional differences and the morphometrical attributes all support the idea that immature sperm, if they are single- or double (most likely triple)-stained may differ in pathogenesis and extent of compensation in the apoptotic process.

We suggest that the data are consistent with the following concepts. When spermatogenesis, spermiogenesis and sperm maturation proceed normally, the process will yield mature fertilizing sperm with a high level of genetic and functional integrity. However, when sperm development becomes faulty, three alternatives may occur. (i) There is early apoptosis in developing germ cells within the adluminal area. These sperm are eliminated and are not present in the ejaculate. (ii) In other immature cells, caspase-3 is activated, and in response Bcl-$_{XL}$ is also expressed which provides a protective effect that substitutes the presence of HspA2. Other immature sperm (caspase-3 only) may survive without any compensatory Bcl-$_{XL}$ expression, most likely because these cells contain HspA2. The continuing development of immature sperm has been observed in the acrosome formation of the HSP70-2 knockout mice in which the meiotic process is interrupted (Mori *et al*., 1999). (iii) In a third type of sperm with diminished maturity that proceeds to elongated spermatids, there are secondary effects of diminished HspA2 chaperone activity, such as cytoplasmic retention, larger and amorphous heads, lack of sperm membrane remodelling and retarded tail sprouting. These immature sperm are more severely affected, and in these 'triple-stained' sperm there is CK retention, in addition to the caspase-3 and Bcl-$_{XL}$ expression. In general, it is unclear what proportion of developing sperm is represented by the apoptotic and immature ejaculated sperm, and what proportion of the total germ cell population is eliminated by apoptosis prior to ejaculation.

In addition to the consistency of the proportions and dimensions of single or double-stained sperm with the CK/caspase-3, caspase-3/Bcl-$_{XL}$ and CK/ combinations, these diminished maturity sperm have all failed to bind to HA. From the point of view of reproduction, this indicates that the CK-, caspase-3- or Bcl-x$_L$-stained immature sperm are non-fertilizing in conventional conception based on sperm–zona pellucida interaction.

Future studies will aim to further define the DNA integrity in the different types of sperm observed. The CK-containing sperm, due to their higher level of lipid peroxidation (Aitken *et al*., 1994; Huszar *et al*., 1994) may contain randomly fragmented DNA, whereas the caspase-3-stained sperm may show DNA degradation with a pattern more closely related to apoptosis. The relative proportions of the subpopulations of mature and diminished maturity sperm in the ejaculate are important in defining why men with similar sperm concentrations have different chances of reproductive success. However, it is clear that not all the immature sperm are eliminated during spermatogenesis and spermiogenesis, and that immature sperm may vary in the type of developmental arrest and in protection mechanisms for apoptosis. These variations are likely to explain the persistence of immature sperm in the ejaculate and contribute to sperm polymorphism.

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References

Aitken RJ, Krausz CS and Buckhinham D (1994) Relationship between biochemical markers for residual cytoplasm, reactive oxygen species generation, and the presence of leukocytes and precursor germ cells in human sperm suspensions. Mol Reprod Dev 39,268–279.

Aitken RJ and Krausz C (2001) Oxidative stress, DNA damage and the Y chromosome. Reproduction 122,497–506.

Beumer TL, Roepers-Gajadien HL, Gademan IS, Lock TM, Kal HB and De Rooij DG (2000) Apoptosis regulation in the testis: involvement of Bcl-2 family members. Mol Reprod Dev 56,353–359.

Blanco-Rodriguez J (1998) A matter of death and life: the significance of germ cell death during spermatogenesis. Int J Androl 21,236–248.

Buzzard KA, Giacca AJ, Killender M and Anderson RL (1998) Heat shock protein 70 modulates pathways of stress-induced apoptosis. J Biol Chem 273,17147–17153.

Celik-Ozenci C, Jakab A, Vigue L, Demir R and Huszar G (2002) Mature and fertile sperm selectively bind to hyaluronic acid: cytoplasmic content, HspA2 levels, chromatin maturity, shape and ICSI sperm selection. J Gynecol Invest 9(49th SGI annual meeting; No. 1, Suppl 849),340A.

Celik-Ozenci C, Catalanotti J, Jakab A, Aksu C, Ward D, Bray-Ward P, Demir R and Huszar G (2003) Human sperm maintain their shape following decondensation and denaturation for FISH: shape analysis and objective morphometry. Biol Reprod 69,1347–1355.

De Kretser DM, Loveland KL, Meihardt A, Simorangkir D and Wreford N (1998) Spermatogenesis. Hum Reprod 13,1–8.

Dix DJ, Allen JW, Collins BW, Mori C, Nakamura N, Poorman-Allen P, Goulding EH and Eddy EM (1996) Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. Proc Natl Acad Sci USA 93,3264–3268.

Eddy EM (1999) Role of heat shock protein HSP70-2 in spermatogenesis. Rev Reprod 4,23–30.

Ergur AR, Dokras A, Giraldo JL, Habana A, Kovanci E and Huszar G (2002) Sperm maturity and treatment choice of in vitro fertilization (IVF) or intracytoplasmic sperm injection: diminished sperm HspA2 chaperone levels predict IVF failure. Fertil Steril 77,910–918.

Gandini L, Lombardo F, Paoli D, Caponecchia L, Familiari G, Verlengia C, Dondero F and Lenzi A (2000) Study of apoptotic DNA fragmentation in human spermatozoa. Hum Reprod 15,830–839.

Garrido C, Gurbuxani S, Ravagnan L and Kroemer G (2001) Heat shock proteins: endogenous modulators of apoptotic cell death. Biochem Biophys Res Commun 286,433–442.

Gergely A, Kovanci E, Senturk L, Cosmi E, Vigue L and Huszar G (1999) Morphometric assessment of mature and diminished-maturity human spermatozoa: sperm regions that reflect differences in maturity. Hum Reprod 14,2007–2014.

Huszar G and Vigue L (1990) Spermatogenesis-related change in the synthesis of the creatine kinase B-type and M-type isoforms in human spermatozoa. Mol Reprod Dev 25,258–262.

Huszar G and Vigue L (1993) Incomplete development of human spermatozoa is associated with increased creatine phosphokinase concentration and abnormal head morphology. Mol Reprod Dev 34,292–298.

Huszar G. and Vigue L (1994) Correlation between the rate of lipid peroxidation and cellular maturity as measured by creatine kinase activity in human spermatozoa. J Androl 15,71–77.

Huszar G, Corrales M and Vigue L (1988) Correlation between sperm creatine phosphokinase activity and sperm concentrations in normospermic and oligospermic men. Gamete Res 19,67–75.

Huszar G, Vigue L and Morshedi M (1992) Sperm creatine phosphokinase M-isoform ratios and fertilizing potential of men: a blinded study of 84 couples treated with in vitro fertilization. Fertil Steril 57,882–888.

Huszar G, Vigue L and Oehninger S (1994) Creatine kinase immunocytochemistry of human sperm-hemizona complexes: selective binding of sperm with mature creatine kinase-staining pattern. Fertil Steril 61,136–142.

Huszar G, Sbracia M, Vigue L, Miller DJ and Shur BD (1997) Sperm plasma membrane remodeling during spermiogenetic maturation in men: relationship among plasma membrane beta 1,4-galactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. Biol Reprod 56,1020–1024.

Huszar G, Stone K, Dix D and Vigue L (2000) Putative creatine kinase M-isoform in human sperm is identified as the 70-kilodalton heat shock protein HspA2. Biol Reprod 63,925–932.

Huszar G, Celik-Ozenci C, Cayli S, Zavaczki Z, Hansch E and Vigue L (2003) Hyaluronic acid binding by human sperm indicates cellular maturity, viability and unreacted acrosomal status. Fertil Steril 79,1616–1624.

Irvine DS, Twigg JP, Gordon EL, Fulton N, Milne PA and Aitken RJ (2000) DNA integrity in human spermatozoa: relationships with semen quality. J Androl 21,33–44.

Kaufmann SH and Hengartner MO (2001) Programmed cell death: alive and well in the new millennium. Trends Cell Biol 11,526–534.

Kovanci E, Kovacs T, Moretti E, Vigue L, Bray-Ward P, Ward DC and Huszar G (2001) FISH assessment of aneuploidy frequencies in mature and immature human spermatozoa classified by the absence or presence of cytoplasmic retention. Hum Reprod 16,1209–1217.

Lalwani S, Sayme N, Vigue L, Corrales M and Huszar G (1996) Biochemical markers of early and late spermatogenesis: relationship between the lactate dehydrogenase-X and creatine kinase-M isoform concentrations in human spermatozoa. Mol Reprod Dev 43,495–502.

Li CY, Lee JS, Ko YG, Kim J and Seo JS (2000) Heat shock protein 70 inhibits apoptosis downstream of cytochrome c release and upstream of caspase-3 activation. J Biol Chem 275,25665–25671.

Manicardi GC, Bianchi PG, Pantano S, Azzoni P, Bizzaro D, Bianchi U and Sakkas D (1995) Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. Biol Reprod 52,864–867.

Manicardi GC, Tombacco A, Bizzaro D, Bianchi U, Bianchi PG and Sakkas D (1998) DNA strand breaks in ejaculated human spermatozoa: comparison of susceptibility to the nick translation and terminal transferase assays. Histochem J 30,33–39.

Mori C, Allen JW, Dix DJ, Nakamura N, Fujioka M, Toshimori K and Eddy EM (1999) Completion of meiosis is not always required for acrosome formation in HSP70-2 null mice. Biol Reprod 61,813–822.

Óvári L, Vigue L, Stronk J, Borsos A, Ward D, Ward P and Huszar G (2003) Detection of numerical chrosomal aberrartions and nuclear immaturity within in the same spermatozoa: a study of FISH and Aniline blue staining. Hum Reprod 18(Suppl 1), p. xviii, 79.

Parcellier A, Gurbuxani S, Schmitt E, Solary E and Garrido C (2003) Heat shock proteins, cellular chaperones that modulate mitochondrial cell death pathways. Biochem Biophys Res Commun 304,505–512.

Print CG and Loveland KL (2000) Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioessays 25,423–430.

Ricci G, Perticarari S, Fragonas E, Giolo E, Canova S, Pozzobon C, Guaschino S and Presani G (2002) Apoptosis in human sperm: its correlation with semen quality and the presence of leukocytes. Hum Reprod 17,2665–2672.

RuckerIII EB, Dierisseau P, Wagner K-U, Garrett L, Wynshaw-Boris A, Flaws JA and Henninghausen L (2000) Bcl-x and BAX regulate mouse primordial germ cell survival and apoptosis during embryogenesis. Mol Endocrinol 14,1038–1052.

Sakkas D, Mariethoz E and St John JC (1999a) Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. Exp Cell Res 251,350–355.

Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG and Bianchi U (1999b) Origin of DNA damage in ejaculated human spermatozoa. Rev Reprod 4,31–37.

Sakkas D, Moffatt O, Manicardi GC, Mariethoz E, Tarozzi N and Bizzaro D (2002) Nature of DNA damage in ejaculated human spermatozoa and the possible involvement of apoptosis. Biol Reprod 66,1061–1067.

Sbracia M, Grasso J, Sayme N, Stronk J and Huszar G (1997) Hyaluronic acid substantially increases the retention of motility in cryopreserved/thawed human spermatozoa. Hum Reprod 12,1949–1954.

Shen HM, Dai J, Chia SE, Lim A and Ong CN (2002) Detection of apoptotic alterations in sperm in subfertile patients and their correlations with sperm quality. Hum Reprod 17,1266–1273.

Steger K, Klonisch T, Gavenis K, Drabent B, Doenecke D and Bergmann M (1998) Expression of mRNA and protein of nucleoproteins during human spermiogenesis. Mol Hum Reprod 4,939–945.

Wei YQ, Zhao X, Kariya Y, Fukata H, Teshigawara K and Uchida A (1994) Induction of apoptosis by quercetin: involvement of heat shock protein. Cancer Res 54,4952–4957.

Wei YQ, Zhao X, Kariya Y, Teshigawara K and Uchida A (1995) Inhibition of proliferation and induction of apoptosis by abrogation of heat-shock protein (HSP) 70 expression in tumor cells. Cancer Immunol Immunother **40**,73–78.

Weng SL, Taylor SL, Morshedi M, Schuffner A, Duran EH, Beebe S and Oehninger S (2002) Caspase activity and apoptotic markers in ejaculated human sperm. Mol Hum Reprod 8,984–991.

World Health Organization (1999) WHO Laboratory Manual for the Examination of Human Semen and Sperm–Cervical Mucus Interaction. 4th edn, Cambridge University Press, Cambridge, UK.

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