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FISH assessment of aneuploidy frequencies in mature and immature human spermatozoa classified by the absence or presence of cytoplasmic retention*

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Previously, a relationship has been found between diminished cellular maturity of human spermatozoa and low-level expression of the testis-specific chaperone protein, HspA2. Because HspA2 is a component of the synaptonemal complex in rodents, and assuming that this is also the case in men, it was postulated that the frequency of chromosomal aneuploidies would be higher in immature versus mature spermatozoa. This question was examined in spermatozoa from semen and from 80% Percoll pellets (enriched for mature spermatozoa) of the same ejaculate in 10 oligozoospermic men. Immature spermatozoa with retained cytoplasm, which signifies spermiogenetic arrest, were identified by immunocytochemistry. Using fluorescence in-situ hybridization (FISH), ~7000 sperm nuclei were evaluated in each of the 20 fractions (142 086 spermatozoa in all) using centromeric probes for the X, Y and 17 chromosomes. The proportions of immature spermatozoa were 45.4 ± 3.4 versus 26.6 ± 2.2% in the two semen versus the Percoll groups (medians: 48.2 versus 25%, $P < 0.001$, $n = 300$ spermatozoa per fraction, total 6000 spermatozoa). There was also a concomitant decline in total disomy, total diploidy and total aneuploidy frequencies in the 80% Percoll versus semen fractions (0.17 versus 0.54%, 0.14 versus 0.26% and 0.31 versus 0.81% respectively, $P < 0.001$ in all comparisons). The mean decline of aneuploidies was 2.7-fold. With regard to the hypothesis that aneuploidies are related to sperm immaturity, there was a close correlation between the incidence of immature spermatozoa and disomies ($r = 0.7$, $P < 0.001$) but no correlation with diploidies ($r = 0.03$), indicating that disomies originate primarily in immature spermatozoa. It is suggested that the common factor underlying sperm immaturity and aneuploidies is the diminished expression of HspA2. In addition, the lack of this chaperone may also cause diminished cellular transport of proteins, such as DNA-repair enzymes or of the retention of cytoplasm that is extruded from normally maturing spermatozoa during spermiogenesis.

Key words: cellular maturity/chromosomal aneuploidy/diploidy frequency variations/HspA2 chaperone protein

Introduction

In an attempt to develop an objective assessment of male fertility, for the past 15 years we have pursued biochemical markers of sperm maturity and function. We have established that semen specimens with high sperm creatine phosphokinase B isoform (CK-B) concentrations have diminished fertility. Further studies showed that this relationship stems from the fact that the high content of CK-B, which is a cytoplasmic enzyme, is a reflection of cytoplasmic retention in spermatozoa, which in turn is caused by incomplete extrusion of the cytoplasm during the last phase of spermiogenesis (Clermont, 1963; Huszar *et al*., 1988; Huszar and Vigue, 1993). Thus, spermatozoa with high CK-B and high cytoplasmic content are of diminished maturity and, consequently, of diminished function. We have shown further that a protein with unique properties, which is thought to be a germ-cell specific CK-M isoform but recently identified as HspA2, a member of the 70 kDa testis-specific chaperone protein family (Huszar *et al*., 2000), is developmentally regulated and synthesized simultaneously with cytoplasmic extrusion. The presence of HspA2 is a characteristic of mature spermatozoa (Huszar and Vigue, 1990). Consequently, in semen samples, the relative concentra-

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tions of the chaperone protein and the CK-B isoform, or chaperone ratio (formerly CK-M ratio), expressed as [% HspA2/(HspA2+CK-B)], reflect the proportion of mature and immature spermatozoa. The negative predictive value of high CK activity and low chaperone ratio for the occurrence of pregnancies in couples treated with intrauterine insemination and IVF (Huszar *et al*., 1990, 1992) was demonstrated. This predictive value is related to the finding that immature spermatozoa with cytoplasmic retention show diminished binding to the zona pellucida (Huszar *et al*., 1994). Further, the proportion of mature and immature spermatozoa shows inter-individual variations even in normal men, and this proportion is largely independent from the sperm concentrations in the ejaculates.

The potential correlation between increased rates of sperm chromosomal abnormalities and male infertility has been explored by various investigators. The advent of fluorescence in-situ hybridization (FISH) with chromosome-specific DNA probes has facilitated the detection of aneuploidies of the X and Y chromosomes and of several autosomes in sperm samples. Most of the publications have focused upon three questions: (i) the rate of aneuploidies in fertile men; (ii) the rate of aneuploidies in infertile men; and (iii) variations in aneuploidy rates of autosomal versus sex chromosomes. An overview of these publications indicates that evidence is inconclusive, and the results show discrepancies (Robbins *et al*., 1993; Williams *et al*., 1993; Meschede *et al*., 1995; Moosani *et al*., 1995; Griffin *et al*., 1996; Downie *et al*., 1997; Bernardini *et al*., 1998; Rubes *et al*., 1998). In normozoospermic human populations the frequency of disomy reported for 10 autosomal chromosomes was a combined 0.13%, while the disomy rate for the sex chromosomes was higher, at 0.43% (0.07% for XX, 0.21% for YY and 0.15% for XY disomies; Spriggs *et al*., 1995; Martin *et al*., 1996). Other studies, however, have found no differences in disomy rates among autosomes and sex chromosomes in normal males (Guttenbach *et al*., 1994; Lu *et al*., 1994; Downie *et al*., 1997). Discrepancies in X and Y disomy rates have also been reported in spermatozoa from oligozoospermic males. Some studies showed no differences between infertile and fertile men: disomy rates for X and Y chromosomes in infertile men were 0.16% and 0.11%, and for the fertile group 0.13% and 0.08% respectively (Miharu *et al*., 1994). Others, however, found increases in selected autosomal and sex chromosome disomies in spermatozoa from infertile versus fertile males, or in men with low and high sperm concentrations (Moosani *et al*., 1995; Finkelstein *et al*., 1998). Based on data using biochemical markers of sperm maturity and function, it is suggested that the casual relationship between aneuploidy rates and infertility is the consequence of an inadequate definition of male infertility. Indeed, this classification is either based on the seminal sperm concentration and motility, or on the fertility history of the couple; whereas in a substantial percentage of oligozoospermic or even normozoospermic men, the proportion of mature spermatozoa will provide a truer indication of sperm fertilizing potential and infertility.

In previous studies, information regarding the relationship between sperm cellular maturity and chromosomal aneuploidies can be obtained from the following. First, the sperm enzyme lactate dehydrogenase C isoform (LDHC4), which is expressed in the developing male germ cell at the time of the commencement of the meiotic process (Wheat and Goldberg, 1977; Salehi-Ashtiani and Goldberg, 1993). It was found that ~40% of men who have an increased proportion of immature spermatozoa with cytoplasmic retention and diminished HspA2 chaperone ratios, also show low sperm LDHC4 concentrations, indicating defects in spermatogenesis that are initiated in the meiotic division stage (Lalwani *et al*., 1996). Second, the fact that the former CK-M was identified as HspA2, the human homologue of the HSP70-2 mouse chaperone protein, is important because HspA2 in human male germ cells is expressed at two critical points: in spermatocytes (although the presence of HspA2 in human synaptonemal complexes is not yet verified), and in terminal spermiogenesis (Huszar *et al*., 1990, 2000). Accordingly, the lack of this protein may be connected to both the defects of the meiotic process and to failure of cytoplasmic extrusion which is, according to our hypothesis, likely to be chaperoned by HspA2. Third, in the rodent model the 70 kDa chaperone forms part of the synaptonemal complex, and in HSP70-2 knock-out mice the meiotic process was disturbed in males (Allen *et al*., 1996; Dix *et al*., 1996). There is good evidence that synaptic anomalies during meiosis, chromosomal abnormalities and male infertility are related (Egozcue *et al*., 1983; MacDonald *et al*., 1994; Martin *et al*., 1996; Vendrell *et al*., 1999).

These observations led us to investigate the incidences of chromosomal aneuploidies in spermatozoa that originate in semen (lower proportion of mature spermatozoa) and in the 80% Percoll density gradient fractions (higher proportion of mature spermatozoa) of the same semen specimens. The enhancement of mature spermatozoa in 80% Percoll fractions is based on the lower specific gravity of immature spermatozoa with cytoplasmic retention compared with mature spermatozoa, which contain only the nucleus, mitochondrion and the sperm membrane (Huszar and Vigue, 1993). The experimental design relied on the observation that both mature and immature spermatozoa are present in virtually all semen samples. Thus, instead of characterizing men as 'oligozoospermic' or 'normozoospermic', or adhering to undefined clinical paradigms of 'fertility' and 'infertility', which may include couples with various origins of infertility, sperm fractions were prepared from the same semen samples containing higher and lower proportions of mature spermatozoa. The differences in constituent mature and immature spermatozoa were assessed by using CK-immunocytochemistry, a marker of cytoplasmic retention. The immature spermatozoa were identified by the presence of retained cytoplasm. Centromeric chromosome probes and FISH were used to detect aneuploidies in the two sperm populations. In line with our hypothesis, in mature and immature spermatozoa there were differences in aneuploidy frequencies, and the proportions of immature spermatozoa with cytoplasmic retention and frequency of aneuploidies were related.

Materials and methods

Preparation of sperm fractions

Semen samples from 10 individuals were utilized. For the study of the semen fractions, 7–10 µl of neat semen were used to prepare each sperm smear on a laboratory glass slide. In order to prepare the corresponding 80% Percoll sperm fraction, an aliquot of the same semen sample was centrifuged through 2.0 ml of an 80% single-phase Percoll gradient at 500 *g* for 20 min at room temperature. The sperm pellet was resuspended in 2 ml human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA, USA) and re-centrifuged at 600 *g* for 10 min in order to eliminate the residual Percoll. The pellet was resuspended in HTF to a concentration of ~30–40$\times 10^6$ spermatozoa/ml, and smears were prepared on glass slides. The smears were fixed with methanol:acetic acid (3:1 ratio) for 10 min, air-dried, dehydrated in a series of 70, 80 and 100% ethanol, and stored at –70° C for the FISH experiments. Other sperm aliquots were subjected to CK-immunocytochemistry in order to determine the proportion of sperm with cytoplasmic retention. For the assessment of aneuploidy frequencies, ~7000 spermatozoa were evaluated in each sample (142 068 sperm nuclei in the 20 fractions of the 10 subjects). For the determination of the proportion of immature spermatozoa, 3×100 spermatozoa were assayed in each fraction (total 6000 spermatozoa). These studies were approved by the Human Investigation Committee of Yale School of Medicine.

CK-immunocytochemistry of individual spermatozoa

The procedures used have been described previously (Huszar and Vigue, 1993; Huszar *et al*., 1994). The washed spermatozoa were allowed to settle onto polylysine-treated microscope slides overnight in a humidity box at 5°C. The overlying solution was carefully pipetted off and replaced by 1% formalin in phosphate buffer/sucrose (PB-suc) for 20 min at 37°C. After removal of the formalin, the slide was allowed to air-dry. The spermatozoa were then blocked with 3% bovine serum albumin in PB-suc at 37°C, and treated with a 1:1000 dilution of polyclonal anti-CK-B antiserum overnight at 4°C (Chemicon Co., Temecula, CA, USA). After further PB-suc washes, the slide was processed with a biotinylated second antibody conjugated with horseradish peroxidase. The brown colour representing the CK-content of spermatozoa was developed by the ABC method (Vector, Burlingame, CA, USA and Sigma, St Louis, MO, USA). On each slide 300 spermatozoa were evaluated by two investigators and characterized as either mature (no cytoplasmic retention) or immature (CK-staining in spermatozoa, indicating cytoplasmic retention) (Figure 1a and b).

Preparation of sperm nuclei

Sperm slides were warmed to room temperature, and in order to render the sperm chromatin accessible to DNA probes, were first treated with 10 mmol/l dithiothreitol (DTT; Sigma) in 0.1 mol/l Tris–HCl, pH 8.0 for 30 min and then with 10 mmol/l lithium diiodosalicylate (LIS; Sigma) in Tris–HCl for 1–3 h.

DNA probes

The FISH studies were carried out using three probes: (i) a 20 kb assigned to the Xp11-Xp21 region of chromosome X (pXBR-1; Yang *et al*., 1982); (ii) Vysis (Downers Grove, IL, USA) alpha satellite rhodamine-labelled probe for the Y chromosome; and (iii) alpha-satellite sequence-specific probe for chromosome 17 (p17H8; Waye and Willard, 1986). The DNA probes for chromosome X and 17 were labelled indirectly with a hapten-conjugated nucleotide (biotin-16-dUTP or digoxigenin-11-dUTP) by nick translation (Rigby *et al*., 1977), and hybridized to metaphase chromosome spreads to develop optimal conditions for probe binding.

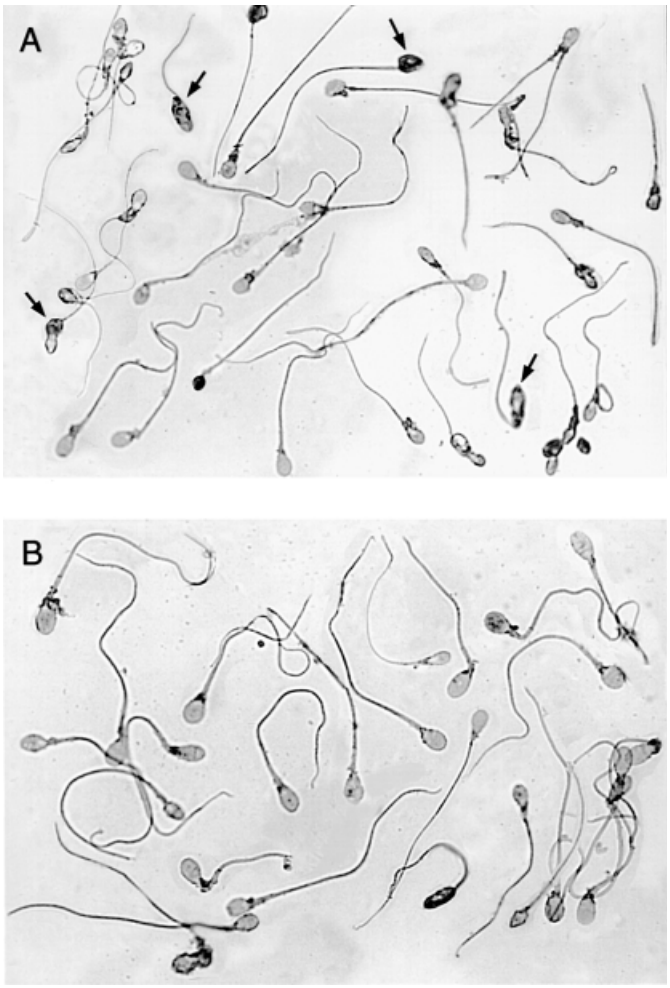

Figure 1. Visualization of the residual cytoplasm in mature and immature spermatozoa by CK-immunochemistry (Huszar and Vigue, 1993; Huszar *et al*., 1994). (**A**) Semen sperm fraction. (**B**) 80% Percoll fraction. Mature spermatozoa have clear heads; immature spermatozoa show various patterns of cytoplasmic retention (arrows). Spermatozoa with cytoplasmic retention are immature, regardless of the extent of the residual cytoplasm (original magnification, ×700).

In-situ hybridization

Since multicolour FISH was necessary for the study of the frequencies of disomy and diploidy in the sex chromosomes, the probes for chromosomes X, Y and 17 were hybridized simultaneously. In these experiments, the chromosome 17 probe was combinatorially labelled with both biotin and digoxigenin nucleotides so that its fluorescence profile would be a combination of red and green (yellow/orange). A 12 µl sample of hybridization mixture (50% formamide, 10% dextran sulphate, 2×SSC) containing the probes was denatured at 75–80°C for 8 min and applied to the slide specimens previously denatured in 70% formamide, 2×SSC for 8 min at 70°C. The hybridization was carried out at 37°C in a moist chamber for 12–24 h.

Post-hybridization washes were performed with 50% formamide-2×SSC three times at 42°C and another three times with 0.1×SSC at 60°C in order to remove excess probe reagents. After a blocking step in 4×SSC/3% bovine serum albumin/0.1% Tween-20 for 30 min at 37°C, the sperm nuclei were incubated for 30 min at 37° C with avidin-FITC (fluorescence green; Roche Biochemicals, Indianapolis, IN, USA) for biotin labelled-probes, and anti-digoxigenin-rhodamine (fluorescence red; Roche Biochemicals) for digoxigenin-labelled

probes. The slides were then washed with 4×SSC/0.1%Tween-20 at 42°C three times, and after staining with 4′-6 diamidino-2-phenylindole (DAPI; Sigma), they were mounted with an antifade solution Vectashield (Vector).

Scoring criteria and data collection

For each patient, two slides of both the initial and the mature sperm fractions were scored by two independent investigators, totalling >14 000 spermatozoa on the four slides. The overall hybridization efficiency in these experiments was >98%. Sperm nuclei were scored according to published criteria (Martin and Rademaker, 1995). Nuclei were eliminated from the scoring if they overlapped, or if they displayed no signal due to hybridization failure. In the case of aneuploidy, the presence of the sperm tail was confirmed. A spermatozoon was considered disomic when it showed two fluorescent domains of the same colour, comparable in size and brightness in the approximately same focal plane, and clearly positioned inside the edge of the sperm head and at least one domain apart. Diploidy was recognized by the presence of two double fluorescence domains with the above criteria. Scoring was performed on an Olympus AX70 epifluorescence microscope primarily with a triple bandpass filter for DAPI, FITC and rhodamine (Chroma Technologies Co., Brattleboro, VT, USA), with monochrome filters for DAPI, FITC and rhodamine for improved signal resolution. Aneuploid spermatozoa were always examined with all of the above filters, and also with a phase-contrast objective in order to verify the presence of the tail and to exclude apparent diploidy in two spermatozoa in close proximity.

Statistical analysis

Statistical analyses were performed using SigmaStat 2.0 (Jandel Corporation, San Rafael, CA, USA). Differences in disomy and diploidy frequencies were analysed using χ^2 analysis of contingency tables. Because of the multiple comparisons in the determination of each nucleus for the various disomies and diploidies, $P \leqslant 0.02$ was considered as the level of significance. Correlation between the proportion of immature spermatozoa and aneuploidy frequencies was examined with Spearman rank correlation.

Results

Semen characteristics, CK-immunocytochemistry and proportion of immature spermatozoa in semen and 80% Percoll sperm fractions

In order to test the hypothesis that aneuploidies are found primarily in immature spermatozoa, 10 moderately oligozoospermic men were studied [mean (± SEM) sperm concentration: 13.3 ± 1.4×10^6 spermatozoa/ml, motility: 50.3 ± 3.4%]. The selection of this patient population was based on studies in which a relationship among cytoplasmic retention, lack of HspA2 expression and sperm immaturity was established. The sperm population from semen contains a higher proportion of immature spermatozoa with brown CK-immunostaining (Figure 1a) than the sperm fraction prepared from the 80% Percoll pellet (Figure 1b), which clearly shows a higher number of spermatozoa with clear heads without cytoplasmic retention. Accordingly, the proportion of immature spermatozoa in the initial semen and 80% Percoll fractions was 45.4 ± 3.4% versus 26.6 ± 2.2% (medians: 48.2 versus 25%, $P < 0.001$, $n = 10$).

XY ratios

Using the probes for the X, Y and 17 chromosomes allowed study of the parameters of X/Y ratio, disomies, diploidies, total disomies, total diploidies and total aneuploidies in the 20 samples (total of 142 068 spermatozoa evaluated). The X/Y ratios were somewhat higher in the 80% Percoll fractions compared with semen, but the differences did not reach significance (1.08 versus 1.05, ranges: 1.0–1.19 versus 0.98–1.08, medians: 0.97 versus 1.12 respectively).

Aneuploidy and diploidy frequencies within and between the semen and 80% Percoll groups

There were substantial differences in aneuploidy and diploidy frequencies between the sperm nuclei arising from semen and from 80% Percoll fractions. Each individual had significant differences (using the stricter level of $P \leqslant 0.02$) between one or more of the aneuploidy and diploidy categories (Table I). Subject #8 had one difference (total disomy), while subjects #5 and #9 had two differences (total disomy and aneuploidy), subjects #3, #4 and #10 had three differences, subjects #1, #2 and #7 had four differences, and subject #6 had five differences in aneuploidy frequencies between their spermatozoa originating in semen and in 80% Percoll. Among the 31 significantly different comparisons within the 10 individuals, 13 were at the level of $P < 0.001$. Twelve of these differences occurred among the disomy comparisons; diploidy XX in subject #2 was the only diploidy difference. The most frequent significantly different comparison between the semen and 80% Percoll sperm fractions in individuals was the total disomy (in all subjects) and total aneuploidy and diploidy (nine of the 10 subjects). Total diploidy frequencies were different only in subjects #6 and #7. With respect to wide variations in disomy and diploidy frequencies within the semen or Percoll fractions among the subjects, there were only two of note: XY disomies (0.03–0.68%, subjects #8 and #1) and XY diploidies (0.01–0.52%, subjects #10 and #5) in the semen fractions. Another aspect to note is the frequency differences in X, Y and XY disomies in spermatozoa of the initial semen (X versus Y: $P = 0.023$; Y versus XY: $P < 0.001$). The cumulative data of the 10 subjects (Table I) indicate that the aneuploidy and diploidy frequencies are significantly lower in the 80% Percoll fractions compared with the semen spermatozoa fractions ($P < 0.001$, $n = 70\,683$ and 71 385 spermatozoa). The exception is YY diploidy.

Analysis of the total disomy, total aneuploidy (disomy + diploidy) categories illustrates well the differences in spermatozoa from semen compared with the 80% Percoll (Table I). There are two major findings: First, there is a significant decline in total disomy (0.54 versus 0.17%), total diploidy (0.26 versus 0.14%) and total disomy and diploidy (0.81 versus 0.31%, $P < 0.001$ in all comparisons). Second, the inter-individual variations in aneuploidy frequencies are also diminished in the 80% Percoll fractions. The distribution of the values is closer, as detected by the ranges of the point plot (Figure 2). These findings are in line with the enhancement of mature spermatozoa in the 80% Percoll fraction. Also, the 80% Percoll fraction is more homogeneous from the point of

Table I. Frequency of the various X, Y and 17 chromosome disomies and diploidies in the semen and 80% Percoll sperm fractions

Subject	Sperm fraction	Disomy X (%)	Disomy Y (%)	Disomy XY (%)	Disomy 17 (%)	XX diploidy (%)	YY diploidy (%)	XY diploidy (%)	Total disomy (%)	Total diploidy (%)	Total aneuploidy (%)
1	Initial	0.11	0.06	**0.68**[c]	**0.17**[a]	0.07	0.04	0.08	**1.02**[c]	0.20	**1.25**[c]
	Percoll 80%	0.03	0.01	**0.13**	**0.03**	0.04	0.00	0.06	**0.20**	0.10	**0.30**
2	Initial	0.04	0.17	0.07	**0.10**[a]	**0.14**[a]	0.07	0.11	**0.38**[a]	0.32	**0.70**[b]
	Percoll 80%	0.03	0.04	0.08	**0.00**	**0.01**	0.07	0.07	**0.16**	0.16	**0.31**
3	Initial	0.01	0.15	**0.27**[c]	0.08	0.06	0.01	0.06	**0.52**[c]	0.13	**0.65**[c]
	Percoll 80%	0.03	0.04	**0.04**	0.03	0.00	0.03	0.03	**0.14**	0.06	**0.20**
4	Initial	0.07	0.17	**0.22**[b]	0.11	0.04	0.10	0.06	**0.57**[c]	0.20	**0.77**[c]
	Percoll 80%	0.03	0.04	**0.06**	0.07	0.01	0.04	0.03	**0.20**	0.08	**0.28**
5	Initial	0.04	0.11	0.24	0.15	0.04	0.06	0.52	**0.55**[b]	0.62	**1.16**[c]
	Percoll 80%	0.04	0.01	0.08	0.08	0.04	0.04	0.32	**0.23**	0.41	**0.64**
6	Initial	**0.17**[a]	0.18	**0.20**[a]	0.11	0.06	0.06	0.17	**0.66**[c]	**0.28**[a]	**0.94**[c]
	Percoll 80%	**0.03**	0.04	**0.04**	0.07	0.00	0.06	0.04	**0.18**	**0.10**	**0.28**
7	Initial	0.11	**0.14**[b]	0.11	0.08	0.06	0.10	0.18	**0.45**[c]	**0.34**[b]	**0.79**[c]
	Percoll 80%	0.03	**0.00**	0.06	0.03	0.01	0.04	0.06	**0.11**	**0.11**	**0.23**
8	Initial	0.07	0.13	0.03	0.06	0.06	0.04	0.11	**0.28**[a]	0.21	0.50
	Percoll 80%	0.01	0.06	0.03	0.00	0.04	0.03	0.10	**0.10**	0.17	0.27
9	Initial	0.08	0.08	0.17	0.07	0.06	0.03	0.22	**0.40**[b]	0.30	**0.70**[b]
	Percoll 80%	0.00	0.04	0.07	0.04	0.03	0.03	0.11	**0.16**	0.17	**0.34**
10	Initial	**0.22**[b]	0.17	0.17	0.06	0.00	0.01	0.01	**0.62**[c]	0.03	**0.64**[b]
	Percoll 80%	**0.06**	0.04	0.10	0.04	0.00	0.00	0.04	**0.24**	0.04	**0.28**
Total	Initial	**0.09**[c]	**0.14**[c]	**0.21**[c]	**0.10**[c]	**0.06**[c]	0.05	**0.15**[c]	**0.54**[c]	**0.26**[c]	**0.81**[c]
	Percoll 80%	**0.03**	**0.03**	**0.07**	**0.04**	**0.02**	0.03	**0.09**	**0.17**	**0.14**	**0.31**

Bold numbers indicate significantly different comparisons, [a]$P < 0.02$, [b]$P < 0.01$, [c]$P < 0.001$ between initial and Percoll 80% fractions.
A total of 142 068 spermatozoa was evaluated (71 385 in the initial semen fraction and 70 683 in the 80% Percoll fraction).

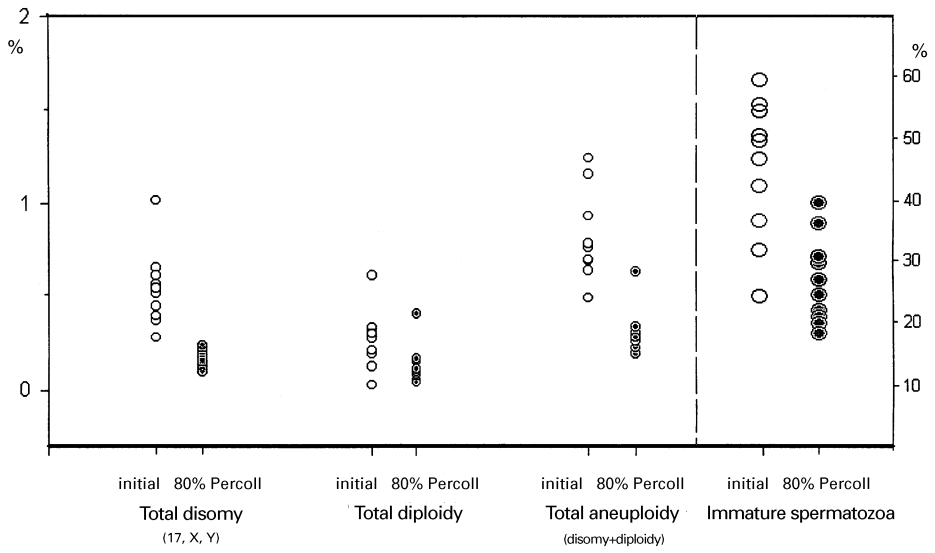

Figure 2. Distribution of aneuploidy frequencies and the proportion of immature spermatozoa in the semen and 80% Percoll fractions in the 10 individuals.

view of sperm maturity, and the aneuploidy and diploidy frequencies are also similar to those in normal men.

The total sperm aneuploidy (disomy+diploidy) frequencies in the initial semen compared with 80% Percoll fractions were reduced 2.7-fold (range: 1.0–6.3). These data do not include the nine instances in which frequencies in the Percoll fractions were reduced to 0% (four disomies and five diploidies). The mean decline in the 10 sample pairs was more distinct in the comparison of disomies (3.2-fold, range: 2.4–5.1) than of diploidies (2.0-fold, range: 0.7 –3.0). Thus, disomies are more related to the elimination of immature spermatozoa from the semen than are diploidies.

Table II. Sperm concentrations and proportions of immature spermatozoa in the 10 semen samples

	Subject										Mean ± SEM
	1	2	3	4	5	6	7	8	9	10	
Sperm concentration ($\times 10^6$/ml)	10	8	12	17	19	19	10	13	15	10	13.3 ± 1.3
Immature spermatozoa (%) in											
Semen	42.0	37.0	47.0	56.0	32.6	59.3	24.6	51.0	49.5	55.5	45.5 ± 3.6
Percoll 80%	28.0	20.0	30.0	23.0	19.3	39.5	21.0	25.0	25.0	36.5	26.7 ± 2.5

Proportions of immature spermatozoa and sperm concentrations in the samples

It has been shown previously that the biochemical parameters of sperm maturity (CK activity, chaperone ratio and proportion of mature/immature spermatozoa) are independent of the sperm concentrations in the samples (Huszar *et al*., 1988, 1990; Huszar and Vigue, 1993). The data of the current study, although the group is small, support this observation well (Table II). If the 10 semen samples are divided according to the five lower and five higher sperm concentrations, the group of five men with lower sperm density (subjects 1, 2, 3, 7 and 10) have an average sperm concentration of $10.0 \pm 0.6 \times 10^6$ spermatozoa/ml, whereas in the other men (subjects 4, 5, 6, 8 and 9) the average is $16.5 \pm 1.2 \times 10^6$ spermatozoa/ml, which is closer to the normozoospermic range (20×10^6 spermatozoa/ml) range. However, the proportion of immature spermatozoa is higher in the group with sperm concentrations of 16.5×10^6 versus 10×10^6/ml (49.8 ± 4.5% versus 41.4 ± 5.2%). This inverse relationship is also evident in the men with the lowest and highest sperm concentrations (subjects 2 versus 5 with 8×10^6 and 19×10^6 spermatozoa/ml), in whom the proportions of immature spermatozoa are 37% and 59% respectively. Finally, the lack of a consistent relationship between sperm maturity and concentrations is best demonstrated by the three men with sperm concentrations of 10×10^6 spermatozoa/ml (subjects 1, 7 and 10). The proportions of immature sperm in these three semen samples are 42, 24 and 55% respectively, bridging the entire range of the 10 men.

Relationship between the proportions of immature spermatozoa and chromosomal aneuploidies

In order to substantiate further a potential relationship between the incidence of immature spermatozoa and of aneuploidies/diploidies, correlation analyses were performed between the proportion of immature spermatozoa with cytoplasmic retention and the frequencies of total disomes, total diploidies and total disomies+diploidies, respectively. In line with our hypothesis, the data indicated that there was a close correlation between the incidence of cytoplasmic retention and disomies in the 20 samples ($r = 0.7$, $P < 0.001$). Among the various disomies, the Y disomy correlated best with the incidence of immature spermatozoa in the samples ($r = 0.78$). However, there was no correlation at all between the immature spermatozoa and the incidence of diploidies ($r = 0.03$). Finally, due to the lack of contribution by the diploidies, there was a less consistent—but still significant—correlation between the incidences of immature spermatozoa and total disomies+diploidies ($r = 0.48$, $P < 0.01$). These data suggest that our hypothesis was correct, and that most of the disomies are found in immature spermatozoa with cytoplasmic retention.

Discussion

Chromosomal aneuploidy, disomy or diploidy occurs when a sperm cell possesses more or less than a single copy of each autosomal or sex chromosome, or more than one copy of the entire genome. As reviewed in the Introduction, the previously reported sperm aneuploidy frequencies between 'fertile' and 'infertile' men are inconsistent, because in some studies too few spermatozoa were evaluated and because normally occurring variations in aneuploidy frequencies among certain chromosomes are not fully recognized (Williams *et al*., 1993; Hassold, 1998; Egozcue *et al*., 2000). Our concepts and data on sperm maturation provides a third line of evidence. We suggest that the primary cause of the variability is the fact that the 'fertile' and 'infertile' designations are based on non-objective criteria, i.e. lack of pregnancies in the couples and/or sperm concentration parameters. In couples with oligozoospermic or asthenozoospermic husbands who otherwise have adequate concentrations of mature and fertile spermatozoa, the wives' ovulatory patterns, tubal patency, antisperm antibody status and similar conditions contributing to infertility are frequently overlooked because of the poor semen parameters and presumed male factor infertility.

The present study was designed to establish the incidence of chromosomal aneuploidies in mature and immature spermatozoa originating in the same semen specimens. Semen and 80% Percoll sperm fractions were studied in which the incidence of immature spermatozoa with cytoplasmic retention, as assessed by CK-immunocytochemistry, was lower. Percoll centrifugation takes advantage of the lower specific gravity of spermatozoa with cytoplasmic retention compared to that of mature spermatozoa (Huszar and Vigue, 1993; Aitken *et al*., 1994). In the 10 subjects we reconfirmed the inter-individual variations in the proportion of mature and immature spermatozoa, independently of their sperm concentrations (Table II). It is of note that in addition to cytoplasmic retention and lack of plasma membrane remodelling, there are also nuclear features of sperm immaturity, such as delay in the histone-protamine transition, or retention of high levels of lysine-rich histones. Indeed, a relationship was reported between aniline blue staining, and the frequency of some chromosomal aneuploidies (Morel *et al.*, 1998).

The goals of this study were as follows: (i) to assess the frequencies of chromosomal aneuploidies in sperm fractions with different proportions of mature and immature spermatozoa; (ii) to examine the relationships between frequencies of either disomy or diploidy and the incidence of immature spermatozoa with cytoplasmic retention; and (iii) to test our hypothesis regarding the potential relationship between sperm immaturity and the incidence of chromosomal aneuploidies, as they may be related to adverse upstream meiotic events of spermatogenesis. We are assuming here that in man (similar to rodents) the 70 kDa HspA2 chaperone is part of the synaptonemal complex. Thus, we anticipated that the common factor leading to increased rates of aneuploidy and arrested spermiogenetic maturation is the diminished expression of the HspA2.

In the mature versus immature sperm fractions there was a somewhat (but not significantly) higher X/Y ratio, very close to the 50–50% range. In the 80% Percoll sperm fractions compared with the semen fractions of individual men (Table I), there was a significant decline in disomy and diploidy frequencies. Among all categories there were 31 such differences, 13 of these were significant at the $P < 0.001$ level, and the others at $P < 0.1$ and $P \leqslant 0.02$. In addition, the man-to-man variation in aneuploidies and in the proportion of the immature spermatozoa also attenuated in the 80% Percoll compared with semen sperm fractions (Figure 2).

With respect to aneuploidy frequencies, it was found that the immature versus mature sperm fractions had substantially higher rates of aberrations, whether considering the X, Y or autosomal chromosome disomies or diploidies. All comparisons were different at the level of $P < 0.001$. The rates for the sex and autosomal chromosome abnormalities were similar, both within the immature (mean of X, Y, XY disomies and diploidies: 0.10%, 17 disomy: 0.10%) and mature spermatozoa (mean of X,Y,XY disomies and diploidies: 0.04%, 17 disomy: 0.04%; Table I), although the XY disomy and XY diploidy rates showed high variability among men. The overall frequencies of disomy plus diploidy provide the aggregate picture. With the exception of the YY diploidies, in which the difference did not reach significance, the other frequencies showed a decline by 1.5- to 4-fold (mean 2.7-fold) in the 80% Percoll fractions. The highest mean aneuploidy rates occurred in the XY disomies. In the 80% Percoll versus the semen fractions, total disomy and total diploidy declined >3-fold and >2-fold respectively (0.54 versus 0.17% and 0.81 versus 0.31%).

In line with our hypothesis, the frequency of aneuploidies and the proportion of immature spermatozoa indicated a significant correlation ($r = 0.48$, $P < 0.001$, $n = 20$ fractions). These data further strengthen the hypothesis that the majority of aneuploidies are found in immature spermatozoa. Further analysis of this correlation yielded a very interesting new finding. The correlation between all disomies and the proportion of immature spermatozoa was $r = 0.70$ (with Y disomies alone, $r = 0.78$), whereas the relationship with diploidies was random ($r < 0.1$). This is in spite of the fact that the diploidy frequencies were lower ($P < 0.001$) in the 80% Percoll compared with the semen sperm fractions. These differences in relationship between immature spermatozoa versus disomies or diploidies indicate there is a higher frequency of impaired meiotic division among immature spermatozoa. However, diploidies—which do not correlate with the incidence of immature spermatozoa in the samples—are likely to arise by diverse cellular mechanisms (Egozcue *et al*., 2000).

Data from other laboratories in men with presumptive mature and diminished maturity sperm populations, in which at least 10 000 nuclei per normozoospermic man were evaluated, showed disomy and diploidy rates which were similar to our data for spermatozoa in the 80% Percoll pellets (Martin *et al*., 1996; Downie *et al*., 1997). For instance, in the former of these studies the mean disomy rates for chromosomes 1, 12, X, Y and XY were 0.07–0.16, while in the latter study the respective values for chromosomes 3, 7, 16, X, Y and XY were between 0.05% and 0.20%, similar to the 0.03% to 0.14% for chromosomes X, Y, XY and 17 in the current study.

Several recent reports have dealt with semen samples of severe oligoozospermia and/or high incidence of abnormal sperm morphology, both of which are indicators of sperm immaturity. In one such study (Storeng *et al*., 1998), aneuploidy rates were studied in 19 men who were triaged to IVF or intracytoplasmic sperm injection (ICSI), based on their higher and lower sperm concentrations. The overall disomy rates, although different in absolute values, were about 20-fold higher in the ICSI group compared with the IVF group. The extremely high rate of aneuploidies in men who have few spermatozoa, and thus are likely to have a high proportion of immature spermatozoa, was also documented (In't Veld *et al*., 1997). According to others (Bernardini *et al*., 1998), in oligoozospermic samples with a high incidence of abnormal sperm morphology, there were significantly higher incidences of sperm aneuploidies. These authors suggest that a direct relationship may exist between the impairment of the spermatogenetic process and increased rates of aneuploidy. This idea accords well with our combined evidence of diminished spermiogenetic maturation and infertility that we developed, based on the high sperm CK content and low levels of HspA2 expression (CK-M at the time) which predicted the lack of pregnancies (Huszar *et al*., 1990, 1992). Further evidence was developed (Aran *et al*., 1999; Pang *et al*., 1999) following investigations of aneuploidy frequencies in male infertility patients. In subjects who had higher sperm density, and presumably higher proportions of mature spermatozoa, there were lower rates of disomies and diploidies compared with ICSI patients. Thus, sperm immaturity is related to meiotic defects indicated by the diminished expression of the HspA2 and LDHC4 (Lalwani *et al*., 1996; Huszar *et al*., 2000), and there was an increased frequency of aneuploidies in men treated with ICSI compared with normal men. There is also an association between sperm immaturity and the increased rates of lipid peroxidation along with the aneuploidies. Heavy smokers, who exhibit antioxidant depletion and increased sperm lipid peroxidation, showed elevated frequencies of disomies as well as an increased proportion of 'round-headed' sperm which, as discussed, is the hallmark of cytoplasmic retention and diminished sperm maturity (Huszar and Vigue,

1993, 1994; Aitken *et al*., 1994; Gomez *et al*., 1996; Rubes *et al*., 1998; Twigg *et al*., 1998).

Considering the clinical aspects of male infertility, an association between oligoozospermia and synaptic anomalies during meiosis has already been suggested in 46,XY males, resulting in aneuploid and diploid spermatozoa (Egozcue *et al*., 1983; Martin *et al*., 1996; Aran *et al*., 1999; Vendrell *et al*., 1999). It is also known that trisomies, originating in non-disjunction during meiosis, are found in ~5% of clinically evidenced pregnancies, although trisomies of paternal origin are rare in newborns and in abortion materials (Hassold *et al*., 1992; MacDonald *et al*., 1994). The variability of non-disjunction rates among men is well demonstrated in the present study by the wide fluctuations in XY disomy frequencies (0.03–0.68%; Table I) among the 10 subjects. Another interesting aspect of the association between poor sperm parameters, reflecting sperm immaturity, and increased frequency of aneuploidies, is the further relationship with abnormal karyotypes (Vegetti *et al*., 2000). Thus, diminished HspA2 concentrations may also cause structural chromosomal aberrations as well as aneuploidies.

The relationship between increased aneuploidy frequencies and sperm immaturity, based on the diminished expression of HspA2, are also supported by a rodent model. Targeted disruption of the *HSP70-2* gene (the homologue of *HspA2* in the mouse) resulted in failed meiosis because HSP70-2 is a component of the mouse synaptonemal complex during the meiotic prophase, and *HSP70-2* disruption results in synaptic abnormalities (Allen *et al*., 1966; Dix *et al*., 1996). The potential connections between aneuploidies and synaptic (meiotic pairing) defects have been suspected earlier (Hultén and Pearson, 1971; Skakkebaek *et al*., 1973; Egozcue *et al*., 1983; Hassold, 1998; Ashley, 2000). Our hypothesis, and the close correlation between disomy and the proportion of immature spermatozoa in the samples ($r = 0.70$–0.78), are consistent with a model in which the diminished presence of HspA2 chaperone in the male germ cell leads to various defects during the meiotic phase and during spermiogenetic maturation (delivery of DNA repair enzymes, cytoplasmic extrusion, plasma membrane remodelling), all of which may be interrupted in the absence of the HspA2. The common origin of defects in both synaptic/meiotic events and sperm maturation is one testable idea, though the alternative possibility that the lack of HspA2 expression and arrested cytoplasmic extrusion and plasma membrane remodelling are corollary consequences of an impaired spermatogenetic programme could also be valid.

In addition to providing a hypothesis for further investigations of the cell biology and the genetic aspects of spermatogenesis, the current study is of particular interest for clinicians who practice ICSI (Palermo *et al*., 1993). Immature spermatozoa, which have a 2- to 4-fold higher rate of chromosome abnormalities than mature spermatozoa (based only on the X, Y and 17 chromosomes), are not likely to be part of the fertilizing pool, because immature spermatozoa that have not completed the spermiogenetic plasma membrane remodelling are deficient in zona-binding site(s) (Huszar *et al.*, 1994, 1997). However, in ICSI, immature spermatozoa may be used for fertilization. One of the consequences is the approximately 4- to 5-fold higher rate of sex chromosome aberrations in offspring of ICSI pregnancies (Bonduelle *et al*., 1998).

In summary, in line with the diminished expression of LDHC4 and HspA2 in immature human spermatozoa, indicating upstream defects of the meiotic process during spermatogenesis (Lalwani *et al*., 1996; Huszar *et al*., 2000), we have now shown that the occurrence of X, Y, XY and 17 chromosome aneuploidies is 1.5- to 4-fold higher in immature compared with mature spermatozoa. Further, a close correlation between the proportion of immature spermatozoa and the frequencies of total aneuploidies and disomies (but not of diploidies) was demonstrated, indicating that disomies primarily occur in immature spermatozoa. Because the proportion of mature spermatozoa show substantial inter-individual variation, independent of sperm concentrations (Huszar *et al*., 1988, 1990, 1992), we suggest that the uncertainty among reports regarding the chromosomal aneuploidy frequencies is due to the fact that infertility is poorly defined by the sperm concentrations, or by the fertility history of couples.

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