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Efficacy of the swim-up method in eliminating sperm with diminished maturity and aneuploidy*

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BACKGROUND: We have previously shown that after 80% Percoll centrifugation there is an overall 2.7-fold reduction of sperm with chromosomal disomies and diploidies (3.2-fold and 2.0-fold respectively), and of sperm with diminished maturity as detected by cytoplasmic retention. The relationship between disomies and immature sperm was $r = 0.7$, suggesting that disomy primarily originates in immature sperm. In the present work we studied the efficacy of the swim-up method in elimination of sperm with diminished maturity and with chromosomal aberrations in the swim-up sperm fractions of 10 patients (sperm concentration: $20 \pm 3.9 \times 10^6$/ml, range 8.9–45.5; sperm motility: 45.2 ± 2.4, all mean ± SEM). METHODS: The validity of the study was enhanced by assessing each sperm fraction with three-colour (X, Y and 17; 5000 sperm) and two-colour (10 and 11; 5000 sperm) chromosome probes using fluorescence in-situ hybridization (FISH). Thus, in each sample 10 000 sperm were evaluated. The incidence of diminished maturity sperm was assessed with creatine kinase immunocytochemistry. RESULTS: In the swim-up fractions there was a reduction in the frequencies of disomic sperm, whether considering the sex chromosomes (1.4-fold) or the three autosomal chromosomes (1.5-fold based on the aggregate frequencies of disomy 10, 11 and 17). There was also a 1.5-fold reduction in diminished maturity sperm, indicating a relationship between the proportion of immature sperm and chromosomal aneuploidies ($r = 0.46$, $P < 0.05$, $n = 20$). Diploid sperm were reduced at a 2.7-fold rate, whether assessed with two- or three-colour FISH. There was a slight increase in the X/Y ratios. CONCLUSIONS: Swim-up reduces the proportion of sperm with chromosomal aberrations and of sperm with diminished maturity. When compared with the results of the previous study with gradient centrifugation performed on semen samples with similar quality, the efficacy after swim-up is lower for disomies and higher for diploidies than that of gradient centrifugation.

Key words: aneuploidy/chromosomal aberrations/diminished maturity/FISH/swim-up

Introduction

The swim-up technique is frequently used for sperm preparation and for an enrichment of the motile sperm fraction in other applications. Previous studies have addressed the variations in motility, morphology and genetic integrity of sperm in swim-up fractions. The question of genetic integrity is particularly important in light of sperm selection for ICSI.

The introduction of fluorescence in-situ hybridization (FISH) with chromosome-specific DNA probes has facilitated the detection of sperm with chromosomal aberrations. Publications dealing with FISH and swim-up have primarily focused upon two questions, without reaching a consensus: (i) what are the X and Y sex ratios in initial semen and swim-up fractions; and (ii) what is the elimination rate of sperm with aneuploidies and diploidies in swim-up fractions. There is substantial diversity in study findings, which may, in part, result from inconsistent patient selection and experimental design. However, the most significant confounding factor is the variation in number of sperm nuclei evaluated. For a reliable assessment of aneuploidy and diploidy rates, considering the mean frequency of 0.1 to 0.5% per chromosome, or one to five aberrant sperm nuclei per 1000, one should evaluate ~5000–10 000 sperm in each sample. In the papers that dealt with sperm swim-up as discussed below, the range of sperm per sample studied was 170–6000. In five of the papers cited, the number of sperm evaluated equalled 1000 or less.

Regarding the X and Y ratios between the initial semen and swim-up sperm fractions, some reports indicated no differences

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(Benet *et al*., 1992; Han *et al*., 1993; Samura *et al*., 1997; Pfeffer *et al*., 1999; Calogero *et al*., 2001). Other authors found an increase in the proportion of Y-bearing sperm after swim-up (Martinez-Pasarell *et al*., 1997; Li and Hoshai, 1998). With respect to elimination of aneuploid sperm, in two studies there were no differences between the initial and swim-up fractions (Samura *et al*., 1997; Van Dyk *et al*., 2000), while others reported a reduction of diploid sperm (Han *et al*., 1993; Martinez-Pasarell *et al*., 1997; Li and Hoshiai, 1998). In comparing sperm selection by three methods, swim-up, glass wool filtration and two-phase discontinuous Percoll gradient centrifugation, Samura *et al*. (1997) found no difference between initial semen and treated sperm fractions with respect to frequencies of diploidy and disomy. The sex chromosome aneuploidy and diploidy rates after swim-up were found to be unchanged by Martinez-Pasarell *et al*. (1997), whereas two other studies indicated a reduction of diploidy frequencies in swim-up fractions (Han *et al*., 1993; Li and Hoshiai, 1998).

There is general agreement that severely oligospermic men, who are candidates for ICSI, have higher rates of aneuploidies than normospermic men (Colombero *et al*., 1999; Pang *et al*., 1999; Zeyneloglu *et al*., 2000; Calogero *et al*., 2001). In two swim-up studies of ICSI patients, the overall aneuploidy frequencies were higher than in normospermic men, but the X/Y ratios and rates of numerical chromosomal abnormalities were similar to those of the initial semen (Benet *et al*., 1992; Calogero *et al*., 2001).

Our interest in the efficacy of the swim-up method in elimination of sperm with aneuploidies and diploidies stems from our previous study, in which we addressed the relationship between the proportions of sperm with diminished cellular maturity and the frequencies of sperm with numerical chromosomal aberrations (Kovanci *et al*., 2001). In that study, as in the present work, immature sperm were monitored by the presence of cytoplasmic retention, which signifies arrest in terminal spermiogenesis, when the surplus cytoplasm of the elongated spermatids is extruded (Clermont, 1963; Huszar *et al*., 1988a; 1990; Huszar and Vigue, 1993). After 80% Percoll gradient centrifugation, the incidences of immature sperm and sperm with aneuploidy and diploidy were reduced in the Percoll pellet compared with the initial semen. The rate of reduction, or clearance, was 3.2× for disomies (range 2.4–5.1× in case of the autosomal- and sex-chromosomes) and 2.0× (range 0.7–3.0×) for diploidy frequencies (Kovanci *et al*., 2001).

There was also a close correlation between the proportion of immature sperm and chromosomal disomes (with an r value of 0.7 for all disomies, and 0.78 for Y disomies, both $P < 0.001$; Kovanci *et al*., 2001). This relationship between the frequencies of chromosomal aneuploidies and diminished sperm maturity is probably based on the finding that in sperm with cytoplasmic retention and diminished maturity, there is a low expression of the 70 kDa testis-specific chaperone protein, HspA2 (Huszar *et al*., 2000). As was shown in the mouse, the HSP70-2, a homologue of the HspA2 chaperone protein, is a component of the synaptonemal complex and also facilitates the intracellular movement of proteins (Dix *et al*., 1996; Eddy, 1999). This association may explain the relationship between meiotic errors, thus aneuploidies, and perhaps the presence of surplus cytoplasm in sperm of diminished maturity. Low sperm HspA2 [previously sperm creatine kinase M subunit (CK-M)] levels were also predictive for failure of pregnancies in two blinded studies of IVF couples (Huszar *et al*., 1992; Ergur *et al*., 2002).

In the present study we examined the efficiency of the swim-up technique in eliminating aneuploid or diploid sperm. In addition to the insight based upon the role of HspA2, the experience with the gradient centrifugation study (Kovanci *et al*., 2001) helped us to an improved experimental design. (i) In general, in samples that are oligospermic or are in the 20–30 × 10^6/ml range there are higher proportions of immature sperm (Huszar *et al*., 1988a; b; 1990; Huszar and Vigue, 1993). For this reason, we have utilized samples primarily with <20 × 10^6sperm/ml, but also have included two normospermic men. (ii) We monitored by CK immunocytochemisty the proportions of sperm with diminished maturity in semen and in the swim-up fractions. (iii) We studied motile sperm yield, or the recovery of motile sperm, in the swim-up fractions. (iv) In order to validate further our study methods and results, we utilized five chromosome probes, and independently employed two- and three-colour FISH in evaluating at least 20 000 sperm in each patient.

Materials and methods

Preparation of sperm fractions by swim-up

Semen samples of 10 individuals, who reported for analysis at The Sperm Physiology Laboratory, Department of Obstetrics and Gynecology, Yale University School of Medicine, were studied. For the studies with FISH or with immunocytochemistry for detection of cytoplasmic retention in sperm, of the initial semen, 7–10 µl of neat semen were used to prepare sperm smears on laboratory glass slides. In order to prepare swim-up sperm fractions we used a home-modified 15 ml Falcon tube, to which 1 ml solid polymer was added; thus the conical sections was filled and the tube provided a flat centrifugation surface (Makler *et al*., 1984). The following procedure is a standard component of our semen analysis, called the 'migration test'. Semen was diluted with human tubal fluid (HTF) medium–0.5% bovine serum albumin in a 1:2 ratio (Irvine Scientific, Santa Ana, CA, USA) and centrifuged at 400 *g* for 10 min. This sediments the motile and non-motile sperm and most cellular components of semen on the flat platform. The supernatant was then carefully discarded with the exception of about 1 ml fluid (this volume facilitates the calculation of the motile sperm yield), and the tube was placed into a 36°C incubator for 30 min. During the incubation, the motile sperm migrated into the supernatant, while the immotile and sluggish sperm and the particulate matter of semen remained at the bottom. After the incubation period, the top 0.5 ml of the supernatant, which is enriched in the motile sperm, was withdrawn carefully and used for the FISH and CK immunocytochemistry experiments. The sperm concentration and motility in the initial and swim-up fractions were determined in a Makler chamber by computer-assisted semen analysis (Hamilton-Thorne Scientific Co., Beverly, MA, USA). All studies were approved by Yale School of Medicine Human Investigation Committee.

Preparation of sperm for the FISH studies

Smears of the initial and swim-up fractions 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 –20°C until FISH was performed.

CK immunochemistry of individual sperm

The procedures used were as described previously (Huszar and Vigue, 1993; Huszar *et al*., 1994; Kovanci *et al*., 2001). The washed sperm (whether from the initial semen or swim-up fractions) were allowed to settle onto polylysine-treated microscope slides overnight in a humidity chamber at 5°C. After a 20 min fixation with 1% formalin at 37°C, the overlying solution was carefully replaced with phosphate buffer/sucrose (PB-suc). The sperm 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). Furthermore, the slide was processed with a biotinylated second antibody conjugated with horseradish peroxidase. The brown colour representing the CK content of sperm was developed by the avidin–biotin complex (ABC) method (Vector, Burlingame, CA, USA and Sigma, St Louis, MO, USA). On each slide 300 sperm were evaluated by two investigators and characterized as either mature (no cytoplasmic retention) or immature (CK staining in sperm, indicating cytoplasmic retention).

Preparation of sperm nuclei for FISH

For decondensation, the sperm slides were warmed to room temperature, and in order to render the sperm chromatin accessible to DNA probes they 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 five probes: (i) a 20 kb repeated family probe assigned to Xp11–Xp21 region of chromosome X (pXBR-1; Yang *et al*., 1982); (ii) microdissected probes for the Y chromosome (Guan *et al*., 1996); alpha-satellite sequence specific centromeric probes for (iii) chromosome 17 (p17H8; Waye and Willard, 1986), (iv) chromosome 10 (pα10RP8; Devilee *et al*., 1988) and (v) chromosome 11 (pLC11A; Waye *et al*., 1987). The DNA probes were labelled indirectly with a hapten-conjugated nucleotide (biotin-11-dUTP for chromosome 10, 17 and X probes, or digoxigenin-11-dUTP for chromosome 11, 17 and Y probes) by nick translation (Rigby *et al*., 1977), and added to metaphase chromosome spreads to develop optimal conditions for hybridization.

FISH

In each individual, the initial and migrated fractions were examined using both two-colour and multicolour FISH. In order to detect the frequency of autosomal disomy and diploidy using chromosome 10 and 11 probes, two-colour FISH was utilized (10–11 assay). Since three probes are necessary to study the frequencies of disomy and diploidy in the sex chromosomes, multicolour FISH was performed when chromosome X, Y and 17 were hybridized together (X-Y-17 assay). In the triple-probe FISH experiments, chromosome 17 was combinatorially detected with both biotin-labelled and digoxigenin-labelled probes, so that its fluorescence profile would be the combination of two colours (in our case red and green resulted in orange/yellow). 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 12–14 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 the 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) 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′ diamino-2-phenylindole (DAPI; Sigma), they were mounted with an antifade solution (Vectashield; Vector Laboratories).

Scoring criteria and data collection

For each patient, two slides (double-probe FISH and triple-probe FISH) of both the initial and the swim-up sperm fractions were scored by two independent investigators, totalling >20 000 sperm 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). Since it is hard to interpret whether an absence of a signal indicates nullisomy or failure of hybridization, nullisomies were disregarded, as per generally accepted methods (Egozcue *et al*., 1997). 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 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 approximately the same focal plane, 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 the triple pass filter for DAPI, FITC and rhodamine (Chroma Technologies Co., Brattleboro, VT, USA), with monochrome filters for DAPI, FITC and rhodamine for improved signal resolution and distinction. If extra intracellular chromosome signal was observed with the triple bandpass filter, it was always examined with the monochrome filters for DAPI (blue only), FITC (green only) and rhodamine (red only) to confirm the existence of an extra chromosome. Aneuploid or diploid sperm were always examined also with a phase-contrast objective in order to verify the presence of the tail and to exclude apparent diploidy in two sperm in close proximity.

For the assessment of aneuploidy frequencies, ~10 000 sperm were evaluated in each sample (207 987 sperm nuclei in the 20 fractions from 10 subjects). For the determination of the proportion of immature sperm, 3× 100 sperm were assayed in each of the 20 samples (a total of 6000 sperm).

Statistical analysis

Statistical analyses were performed using SigmaStat 2.0 (Jandel Corporation, San Rafael, CA, USA). Differences in disomy and diploidy frequencies, as well as immature sperm rates were analysed using the χ^2 analysis of contingency tables. Mann–Whitney rank sum test were used to analyse the motility differences between the fractions. Correlations between the motility, the proportion of immature sperm and aneuploidy frequencies were examined with the Pearson correlation test.

Results

Sperm concentration, motility and cellular maturity in semen and swim-up fractions

We studied 10 men with mean (± SEM) sperm concentrations of $20.2 \pm 3.9 \times 10^6$ sperm/ml semen (range 8.9–45.5; Table I).

Table I. Characteristics of the semen and swim-up fractions

Man	Fraction	Concentration ($\times 10^6$/ml)	Motility[a] (%)	Motile sperm yield[b] (%)	Diminished maturity sperm (%)	Clearance of diminished maturity sperm ($\times$ reduction)[c]	*P*
1	**Initial**	10	50.0		51.7		
	Swim-up		90.0	52.0	41.0	1.3	0.025
2	**Initial**	45.5	42.1		64.3		
	Swim-up		92.6	43.4	35.7	1.8	<0.001
3	**Initial**	22.3	46.1		29.0		
	Swim-up		76.1	49.5	22.0	1.3	NS
4	**Initial**	12.2	30.7		43.0		
	Swim-up		30.0	10.0	38.0	1.1	NS
5	**Initial**	32.7	48.4		21.0		
	Swim-up		90.0	70.8	13.0	1.6	0.011
6	**Initial**	15.8	42.3		42.0		
	Swim-up		75.0	51.5	29.0	1.4	<0.001
7	**Initial**	10	43.4		62.0		
	Swim-up		70.0	15.5	51.3	1.2	NS
8	**Initial**	8.9	59.2		47.7		
	Swim-up		50.0	6.6	19.7	2.4	<0.001
9	**Initial**	15	50.0		36.5		
	Swim-up		87.8	67.1	18.7	2.0	<0.001
10	**Initial**	30	40.0		46.7		
	Swim-up		80.0	75.0	27.7	1.7	<0.001
Total	**Initial**[d]	20.2 (3.9)	45.2 (2.4)		44.4 (4.3)		
	Swim-up[d]		74.2 (6.3)	44.1 (8.0)	29.6 (3.7)	1.5 (0.1)	<0.001

[a]Module sperm are defined as curvilinear velocity >7.0 μm/s.
[b]Motile sperm yield defined as proportion of motile sperm from the initial sample recovered in the swim-up.
[c]Clearance derived from initial diminished maturity/swim-up.
[d]Mean (SEM).

We used three samples each from the <10 and the 10–20 $\times$ 10^6 sperm/ml concentration ranges, two samples from the 20–30 $\times$ 10^6 sperm/ml range and two samples from the >30 $\times$ 10^6 sperm/ml range. The selection of these groups reflects our intent to include patients with differing proportions of mature and diminished maturity sperm, and thus different levels of chromosomal aneuploidies. Sperm motility (motile sperm are defined as curvilinear velocity >7.0 μm/s) in the swim-up fractions versus the initial semen was significantly higher (74.2 $\pm$ 6.3 versus 45.2 $\pm$ 2.4%; $P = 0.005$, $n = 10$ pairs). The motile sperm concentration in the 10 initial samples was 8.8 $\pm$ 1.2 $\times$ 10^6 motile sperm/ml. The efficiency of the swim-up procedure was measured by the motile sperm yield (proportion of motile sperm from the initial semen recovered in the swim-up fraction). Motile sperm yield in this group was 44% (Table I). Along with increases in the percentage of motile sperm, the proportion of sperm with diminished maturity (reflected by CK immunostaining, which highlights cytoplasmic retention) declined in the swim-up fractions compared with initial semen (29.6 $\pm$ 3.7 versus 44.4 $\pm$ 4.3%; $P < 0.001$), with an overall reduction rate of 1.5.

XY ratios

In the assessment of two independent aliquots from each sperm fraction, we used probes for the X, Y and 17 chromosomes, and for the 10 and 11 chromosomes, scoring ~105 000 and ~103 000 sperm nuclei, respectively (Table II). Although the X/Y ratios were close to 1:1, the swim-up fractions showed ratios consistently lower than those of initial semen fractions, with the exception of samples from patient 1. Regarding the increase in the proportion of Y-bearing sperm, significance was reached only in the samples of patients 9 and 10. However, after totalling counts, the group of 10 patients showed a difference at the $P < 0.001$ level (means: 1.09 versus 1.04; medians: 1.10 versus 1.05).

Disomy frequencies in the semen and swim-up sperm fractions

A variation in disomy frequencies was observed among the patients, as well as among the five chromosomes evaluated (Table II). The mean aggregate disomy frequencies for X, Y and 17 chromosomes in initial semen and swim-up fractions were 0.32 and 0.21%, a 1.5$\times$ reduction ($P < 0.001$). The mean aggregate disomy frequencies with probes for autosomes 10 and 11 were very similar to those for X, Y and 17 at 0.29 and 0.21%, respectively, an ~1.4-fold reduction ($P < 0.01$). The mean frequencies of disomies were different within patients. For instance, the ranges of X, Y and 17 disomies were 0.12–0.64% in the initial semen and 0.08–0.33% in the swim-up fractions. It is of interest that proportions of the different sex chromosomal disomies were not evenly distributed among the men. The prevailing disomy in the initial semen was XY in patients 1, 3 and 5, while X disomy was predominant in patients 4, 8 and 10, and Y disomy was most prevalent in patient 9.

There was no relationship between sperm concentrations and frequencies of aneuploidies. For instance, the five men in the upper sperm concentration range (patients 2, 3, 5, 6 and 10) and the five men in the lower sperm concentration range (patients 1,

Table II. Disomy frequencies and sex ratio in the semen and swim-up fractions determined by using probes for chromosomes 10, 11, 17, X and Y

Man	Fraction	Probes 17, X and Y (three-colour FISH)								Probes 10 and 11 (two-colour FISH)			
		No. sperm evaluated	X/Y ratio	Disomy X (%)	Disomy Y (%)	Disomy XY (%)	Sex disomy (%)	Disomy 17 (%)	Aggregate disomy (sex+17) (%)	No. sperm evaluated	Disomy 10 (%)	Disomy 11 (%)	Aggregate disomy (10+11) (%)
1	**Initial**	5281	1.09	0.04	0.04	0.11	0.19	0.06	0.25	5056	0.06	0.20	0.26
	Swim-up	5015	1.10	0.04	0.04	0.04	0.12	0.04	0.16	4827	0.17	0.21	0.37
2	**Initial**	7336	1.05	0.08	0.07	0.08	0.23	0.08	0.31	5147	0.33	0.18	0.51
	Swim-up	7009	1.01	0.06	0.07	0.07	0.20	0.04	0.24	5117	0.14	0.20	0.33
3	**Initial**	5032	1.12	0.08	0.02	0.10	0.20	0.14	0.34	5160	0.17	0.14	0.31
	Swim-up	5012	1.08	0.02	0.06	0.14	0.22	0.04	0.26	5021	0.06	0.10	0.16
4	**Initial**	5071	1.04	0.12	0.04	0.08	0.24	0.10	0.34	5056	0.12	0.06	0.18
	Swim-up	4872	1.03	0.06	0.00	0.08	0.14	0.02	0.16	5056	0.04	0.08	0.12
5	**Initial**	5054	1.05	0.02	0.04	0.06	0.12	0.00	0.12	5022	0.10	0.12	0.22
	Swim-up	5030	1.00	0.04	0.02	0.06	0.12	0.02	0.14	5040	0.06	0.08	0.14
6	**Initial**	5146	1.06	0.06	0.06	0.18	0.29	0.35	**0.64***	5104	0.14	0.35	0.49
	Swim-up	5087	1.05	0.04	0.02	0.08	0.14	0.20	**0.33***	5082	0.06	0.20	0.26
7	**Initial**	5128	1.11	0.06	0.06	0.04	0.16	0.18	0.33	5104	0.06	0.22	0.28
	Swim-up	5101	1.07	0.06	0.04	0.02	0.12	0.08	0.20	5072	0.00	0.22	0.22
8	**Initial**	5350	1.18	0.11	0.02	0.04	0.17	0.08	0.24	5356	0.06	0.17	0.22
	Swim-up	5219	1.10	0.04	0.02	0.00	0.06	0.02	0.08	5134	0.00	0.14	0.14
9	**Initial**	5073	**1.14***	0.08	0.14	0.06	0.28	0.12	0.39	5284	0.10	0.17	0.27
	Swim-up	5049	**1.04***	0.08	0.12	0.02	0.22	0.10	0.32	5213	0.04	0.13	0.17
10	**Initial**	5030	**1.10****	0.08	0.04	0.02	0.14	0.12	0.26	5129	0.10	0.12	0.21
	Swim-up	5047	**0.94****	0.08	0.00	0.04	0.12	0.08	0.20	5065	0.08	0.08	0.16
Total	**Initial**	53 501	**1.09*****	0.07	0.05	0.08	**0.20****	0.12	**0.32*****	51 418	0.12	0.17	**0.29****
	Swim-up	52 441	**1.04*****	0.05	0.04	0.06	**0.15****	0.06	**0.21*****	50 627	0.07	0.14	**0.21****
	Clearance			1.4	1.3	1.4	1.4	1.9	1.5		1.8	1.2	1.4

Bold numbers represent significantly different comparisons: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

4, 7, 8 and 9) had disomy frequencies of 0.33% (range 0.12–0.64) and 0.31% (range 0.25–0.39). This finding is in agreement with our previous studies, which demonstrated that both the proportions of immature sperm and the frequencies of aneuploidies were independent of sperm concentrations in semen samples (Huszar *et al*., 1988a; 1990; 1992; Kovanci *et al*., 2001). However, in line with the association between diminished sperm maturity and the frequencies of aneuploidies, there was a moderate correlation between the incidences of sperm with cytoplasmic retention and disomies in the initial semen and in the swim-up fractions ($r = 0.46$, $P < 0.05$, $n = 20$).

Although disomy frequencies showed a declining trend in the swim-up fractions, these changes did not reach statistical significance for any of the five chromosomes investigated, except in one patient (no. 6). In this patient, the total frequency of 17, X and Y chromosome disomies was significantly higher in semen and lower in swim-up fraction (0.64 versus 0.34%; $P < 0.05$). Considering the data from all five probes following the swim-up procedure, the overall disomy frequencies decreased from 0.61% (range 0.34–1.13%) to 0.42% (range 0.21–0.59), with a reduction rate of 1.5×. In spite of variations in aneuploidy frequencies among the chromosomes studied, the reductions of the various disomies were proportional, as indicated by the correlations between aneuploidy frequencies in the swim-up versus initial semen fractions for Y disomy ($r = 0.75$, $P < 0.01$), 11 disomy ($r = 0.76$, $P = 0.01$), 17 disomy ($r = 0.9$, $P < 0.001$) and all five disomies ($r = 0.75$, $P = 0.01$). Conversely, the motile sperm yield was related to the clearance factor of eliminating disomies from the swim-up fractions ($r = 0.65$, $P < 0.05$, $n = 10$).

Diploidy frequencies in sperm of semen and swim-up fractions

The diploidy frequencies and reductions in the swim-up fractions showed outcomes quite different from those of the disomies (Table III). With respect to the three-colour FISH approach, there was a significant reduction in six of the 10 patients, with a 3-fold decline in diploidy rates for the group of 10 patients. Similarly, with probes 10 and 11, there was a significant decline in six of the 10 patients. Thus, the decline in diploidy frequencies was statistically significant in eight of the 10 samples at the level of $P < 0.01$. Five of these men showed declines with both the two- and three-colour FISH probes, whereas diploidy frequencies with the X, Y and 17 probes were reduced in patient 2, and with the two-colour FISH in patient 1. There was also considerable inter-individual variations, with a mean 0.65% (range 0.13–1.76%) in the initial semen, which decreased significantly to 0.24% (0.03–0.79%) in the swim-up fractions ($P < 0.001$). This decline represents a 2.7-fold reduction rate for diploidies.

Diploidy frequencies with three-colour and two-colour FISH, which represent independent measures in the various samples, showed a correlation of $r = 0.99$ ($P < 0.001$, $n = 10$) for the initial semen fractions. In the swim-up fractions, the correlation was a comparable ($r = 0.89$, $P < 0.001$, $n = 10$). The inter-assay correlation of the diploidy frequencies between the three-colour and two-colour FISH was also comparable ($r = 0.99$, $P < 0.001$, $n = 20$). These relationships were valid for diploidies detected by all chromosome probes and in all 10 men, as indicated by the close correlation between the reductions of chromosomal aberrations in the semen and

Table III. Diploidy frequencies in the semen and swim-up fractions

Man	Fraction	Probes 17, X and Y (three-colour FISH)		Probes 10 and 11 (two-colour FISH)		All diploidies detected	
		No. sperm evaluated	Diploidy (%)	No. sperm evaluated	Diploidy (%)	No. sperm evaluated	Diploidy (%)
1	**Initial**	5281	0.21	5056	**0.30**[a]	10 337	**0.25**[b]
	Swim-up	5015	0.06	4827	**0.08**[a]	9842	**0.07**[b]
2	**Initial**	7336	**0.16**[c]	5147	0.18	12 483	**0.17**[d]
	Swim-up	7009	**0.03**[c]	5117	0.04	12 126	**0.03**[d]
3	**Initial**	5032	0.42	5160	0.35	10 192	**0.38**[e]
	Swim-up	5012	0.20	5021	0.16	10 033	**0.18**[e]
4	**Initial**	5071	0.41	5056	0.32	10 127	0.36
	Swim-up	4872	0.21	5056	0.24	9928	0.22
5	**Initial**	5054	0.16	5022	0.10	10 076	0.13
	Swim-up	5030	0.04	5040	0.06	10 070	0.05
6	**Initial**	5146	**1.59**[f]	5104	**1.92**[f]	10 250	**1.76**[f]
	Swim-up	5087	**0.57**[f]	5082	**0.67**[f]	10 169	**0.62**[f]
7	**Initial**	5128	**1.40**[f]	5104	**1.72**[b]	10 232	**1.56**[f]
	Swim-up	5101	**0.49**[f]	5072	**1.08**[b]	10 173	**0.79**[f]
8	**Initial**	5350	**0.95**[f]	5356	**1.20**[f]	10 706	**1.07**[f]
	Swim-up	5219	**0.27**[f]	5134	**0.21**[f]	10 353	**0.24**[f]
9	**Initial**	5073	**0.47**[g]	5284	**0.65**[f]	10 357	**0.56**[f]
	Swim-up	5049	**0.12** [g]	5213	**0.10**[f]	10 262	**0.11**[f]
10	**Initial**	5030	**0.28**[e]	5129	**0.29**[a]	10 159	**0.29**[f]
	Swim-up	5047	**0.06**[e]	5065	**0.08**[a]	10 112	**0.07**[f]
Total	**Initial**	53 501	**0.59**[f]	51 418	**0.70**[f]	104 919	**0.65**[f]
	Swim-up	52 441	**0.20**[f]	50 627	**0.27**[f]	103 068	**0.24**[f]
	Clearance		3.0		2.6		2.7

Interassay correlation $r = 0.992$ ($P < 0.001$, $n = 20$) (initial $r = 0.992$, $n = 10$, swim-up $r = 0.893$, $n = 10$).
Correlation between diploid frequency in initial versus swim-up: $r = 0.925$ ($P < 0.001$, $n = 10$).
Bold numbers represent significantly different comparisons: [a]$P < 0.05$; [b]$P < 0.01$; [c]$P = 0.02$; [d]$P = 0.002$; [e]$P < 0.02$; [f]$P < 0.001$; [g]$P = 0.002$.

swim-up fractions ($r = 0.93$, $P < 0.001$). These data suggest a general clearance factor specific for all diploid sperm: most likely the bulky head of diploid sperm hinders progressive motility.

Discussion

In the present work we studied the frequencies of numerical chromosomal aberrations and incidences of sperm with diminished maturity in both initial semen and swim-up sperm fractions. We wished to re-examine the efficiency of swim-up preparations in elimination of sperm with numerical chromosomal aberrations. Based on the data, we were also interested in comparing the efficacy of swim-up and density gradient centrifugation approaches in the elimination of sperm with diminished maturity (Kovanci *et al*., 2001).

We optimized the experimental design of the swim-up studies based on our experience with gradient centrifugation experiments. (i) In general, men having oligospermia or low normal sperm concentrations have a higher proportion of sperm with diminished maturity. Thus, we primarily used semen samples with sperm concentrations around the 20×10^6 sperm/ml range. However, since swim-up separation required a sufficient number of motile sperm, this precluded the use of severely oligospermic and/or asthenospermic samples. (ii) We monitored 'motile sperm yield', a parameter that reflects recovery of motile sperm in the swim-up fraction. The motile sperm yield is related to the clearance rate of disomic sperm ($r = 0.65$, $P < 0.001$, $n = 10$). (iii) We used five different chromosomal probes on two independent slides: X, Y and 17 as three-colour FISH, and 10 and 11 as two-colour FISH (10 000 sperm in each fraction and 20 000 sperm in each man evaluated). Our results were further validated by the correlation of $r > 0.9$ between the comparable data with three-colour and two-colour FISH. (iv) Finally, in addition to monitoring chromosomal aberrations, we also assessed the proportions of immature sperm both in initial semen and in the swim-up fractions. Sperm with diminished maturity were identified by the presence of retained cytoplasm, as highlighted by CK immunocytochemistry. The proportions of sperm with diminished maturity and with chromosomal aberrations showed a correlation ($r = 0.46$, $P < 0.05$, $n = 20$). This correlation was not as close as that for the gradient centrifugation study, in which the proportion of immature sperm and the frequencies of sperm with disomies were correlated at $r = 0.7$ for all disomies, and $r = 0.78$ for the Y disomies (Kovanci *et al*., 2001). This difference indicates that sperm motility is less discriminatory than sperm density and buoyancy, which are the bases of the gradient fractionation.

In comparing the efficiency of the swim-up and gradient methods in eliminating aneuploid and diploid sperm, one has to consider whether, despite moderate differences in actual sperm concentrations and motilities (20.3 ± 3.8 versus $13.3 \pm 1.4 \times 10^6$ sperm/ml, 45.2 ± 2.4 versus $50.3 \pm 3.4\%$, respectively), the two study populations are similar. We suggest that this is the case, and this notion is supported by three factors. (i) The motile sperm concentrations, which are the important determinants regarding the efficiency of sperm separation by either swim-up or gradient centrifugation, are similar in the two groups (8.8 ± 1.6 versus $6.7 \pm 0.8 \times 10^6$

sperm/ml; P = not significant; medians: 6.0 and 6.6, respectively). (ii) The proportions of diminished maturity sperm, which are related to the frequencies of aneuploidies, are virtually identical in the initial semen samples (45.5 ± 3.6 versus 44.4 ± 4.3%). (iii) The third argument, that sperm concentrations are not a reliable measure of sperm quality and maturity, is based on the various biochemical marker experiments, and is evident in the present study (see inconsistencies between sperm concentrations and percentage diminished maturity sperm in patients 2 and 10, or patients 7 and 8; Table I).

The relationships between diminished sperm maturity and chromosomal disomies and diploidies may be explained by the roles of the HspA2 chaperone in both supporting meiosis as a component of the synaptonemal complex, and in facilitating cellular movement of proteins, a function that we believe may involve cytoplasmic extrusion (Eddy, 1999; Huszar *et al*., 2000). Thus, sperm with diminished maturity and low HspA2 expression level may show both increased frequencies of meiotic errors, causing numerical chromosomal aberrations and cytoplasmic retention, which in turn affect shape and density of sperm (due to the fact that the excess cytoplasm is lighter than DNA and the nuclear components). Relationships between synaptic anomalies during meiosis, chromosomal abnormalities and male infertility were recognized earlier (Egozcue *et al*., 1983; Vendrell *et al*., 1999).

Our data indicate that the swim-up step eliminates sperm with disomies and diploidies, and sperm with diminished maturity with an overall significant reduction at the level of $P < 0.001$. However, the results were not consistent. Of the 10 patients there were seven that reached significant declines in proportions of immature sperm, and only one in the proportion of disomic sperm (Tables I and II). Regarding diploidies, six of the 10 men reached a significant reduction in the swim up fraction, and this pattern was similar whether we considered data from three- or two-colour FISH (Table III). This outcome is different from that reported in the previous gradient centrifugation study, in which all 10 patients showed a decline in disomy frequencies, and only two in diploidy frequencies in the Percoll pellet versus semen sperm fractions (Kovanci *et al*., 2001). In addition, the difference between the present swim-up study and the gradient centrifugation approach was evident considering the clearance rates for disomic sperm of 1.5–1.4-fold (the three- and two-colour FISH) and 3.2-fold, respectively. It is of further interest that diploid sperm showed much higher rates of clearance with swim-up (2.7-fold) compared with those for disomic sperm (1.5–1.4-fold). In line with our swim-up results in a recent study, utilizing swim-up fractionation of sperm, a reduction in frequencies of disomies and diploidies with a clearance rate of ~1.5-fold was reported (Ong *et al*., 2002).

The higher efficiency of gradient centrifugation versus swim-up is due to the fact that, in the Percoll gradient, sperm with cytoplasmic retention do not reach the pellet, whereas, in swim-up fractionation, the differing swimming efficiency of the mature versus diminished maturity sperm (particularly diploid sperm), by virtue of sperm head shape and swimming pattern, is a likely contributory factor. Differences in sperm velocity between sperm with normal and abnormal morphology have been recognized previously (Katz *et al*., 1985). We have found previously that cytoplasmic retention, as evidenced by CK immunocytochemistry, is related to abaxial insertion of the tail, a larger and rounder sperm head size and to an increased proportion of amorphous sperm heads (Huszar and Vigue, 1993). Furthermore, in a study of objective sperm morphometry, sperm midpiece shape, tail length and the ratio of tail length/large head axis were directly related to CK activity and HspA2 ratios within sperm fractions (Gergely *et al*., 1999). Further supporting evidence for the relationship between sperm immaturity, sperm shape and chromosomal aneuploidies was provided by the demonstration of increased frequencies of sperm disomy and diploidy in teratozoospermic men (Harkonen *et al*., 2001).

In addition to cytoplasmic retention as indicative of the relationship between HspA2 expression and sperm maturity, nuclear attributes such as aniline blue staining, which is a marker of persisting histones in immature sperm, have been explored previously. Selection of mature sperm, by binding to immobilized hyaluronan, eliminated sperm cells with aniline blue staining or with cytoplasmic retention (Huszar *et al*., 2003). This result is in agreement with an earlier study, in which a relationship was found between frequencies of sperm with disomies and aniline blue staining in semen samples (Morel *et al*., 1998).

Regarding assisted reproduction, the presence of aneuploid sperm with diminished levels of plasma membrane remodelling and zona-binding sites in sperm preparations is not an important problem with conventional fertilization via IUI or IVF, since these sperm also have diminished fertilizing capacity (Huszar *et al*., 1997). However, the issue has became prominent with the introduction of ICSI, in which the zona pellucida selection barrier is overridden upon fertilization. The power of zona sperm selection is well demonstrated by a small scale study (500 sperm per slide scored), in which disomy rates in sperm from men treated with ICSI were determined both in the swim-up and hemizona-bound sperm fractions. As one might expect, based on the relationship among HspA2 expression, sperm membrane remodelling and formation of the zona-binding site(s), the combined aneuploidy frequency for 18, X, Y and XY disomies was ~1.1% in semen and in the swim-up fractions, whereas in hemizona-bound sperm, the rates were <0.4% (Huszar *et al*., 1997; 2000; Van Dyk *et al*., 2000).

In summary, we have found that swim-up fractionation results in a reduction in sperm having disomies and diploidies and in sperm with diminished maturity. Our work using gradient centrifugation (Kovanci *et al*., 2001) showed an even more efficient elimination of disomic sperm. Gradient centrifugation, in which density of the sperm cell is the major factor, is very efficient in reducing sperm with disomies, but is not as efficient for eliminating diploidies. On the other hand, the swim-up method is very efficient in reducing the proportion of diploid sperm: the large-headed diploid sperm remain preferentially in the lower phase, due to their swimming inefficiency. In addition to providing additional experimental support for the efficacy of swim-up, the present results, based on the assessment of 200 000 sperm, have resolved the

inconsistencies reported in the earlier publications that are reviewed in the Introduction. Our data suggest that the discrepancies were primarily related to inadequate numbers of sperm nuclei examined.

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