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Morphometric assessment of mature and diminished-maturity human spermatozoa: sperm regions that reflect differences in maturity*

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As part of our studies on sperm maturity and function, we examined the head, midpiece and tail of human spermatozoa using computerized morphometry in order to determine which regions reflect the differences between mature spermatozoa and spermatozoa of diminished cellular maturity. We studied 20 men, who were divided into two groups based on their lower (LCKM: 14.6 ± 7.0%, $n = 8$) and higher sperm creatine kinase (CK-M) isoform ratios (HCKM: 48.0 ± 4.3%, $n = 12$) in the initial semen. Using a sequential centrifugation method which relies on the lower density of immature spermatozoa with retained extra cytoplasm, we prepared three sperm fractions with progressively declining maturity, as confirmed with CK-M isoform ratio measurements. Following the sequential fractionation, we affixed the spermatozoa to glass slides, stained the midpiece and the sperm contour, and photographed 25 spermatozoa in each of the 60 fractions (1509 spermatozoa in all). The spermatozoa were then individually digitized on the Image-1 system, and the dimensions of the head, midpiece, and tail were determined. While the data showed significant differences in the midpiece and tail dimensions between the mature and diminished-maturity sperm fractions, the head dimensions were similar and did not reflect sperm maturity. We postulated that the relationship between the biochemical markers of sperm maturity and sperm morphology is based on common spermiogenic events. The data support this idea. In immature spermatozoa in which cytoplasmic extrusion, CK-M isoform expression, and tail sprouting are all diminished, the retained extra cytoplasm in the midpiece and shorter tail length contribute to the morphological variations that we identified by morphometry and considered in sperm morphology. These morphometric features, in association with fluorochrome-coupled biochemical probes, can facilitate the identification of mature spermatozoa in computer-assisted semen analysis.

Key words: biochemical markers/creatine kinase-M/fertility/spermiogenesis/tail length

Introduction

The focus of our research in the past few years has been the objective assessment of male fertility using the biochemical markers of sperm function. We initially found that various semen samples displayed higher and lower levels of sperm creatine kinase (CK) activity (Huszar *et al*., 1988a,b). Through CK immunocytochemistry of individual spermatozoa, we showed that the increased CK activity in immature spermatozoa was a consequence of higher levels of retained cytoplasm (Huszar and Vigue, 1993). We postulated that increased cytoplasmic retention signified an interruption of spermiogenesis and diminished sperm maturity. In spermatozoa that had completed cytoplasmic extrusion and showed low CK activity, we identified a new sperm CK isoform (in addition to the CK-B) with electrophoretic properties similar to those of the muscle CK-M isoform. The proportion of the CK-M and CK-B thus predicted the proportions of mature and immature spermatozoa in the samples (Huszar and Vigue, 1990). In further work, we have demonstrated that sperm CK activity and the CK-M isoform ratio [%CK-M/(CK-M+CK-B)] were closely related and predicted the occurrence of pregnancy or lack of pregnancy in couples treated with intrauterine insemination or with in-vitro fertilization (IVF) (Huszar and Vigue, 1990; Huszar *et al*., 1990, 1992). The explanation for this predictive value was established in studies demonstrating that immature spermatozoa with cytoplasmic retention failed to bind to the zona pellucida of human oocytes (Huszar *et al*., 1994). This suggested to us that, simultaneously with the spermiogenic events of cytoplasmic extrusion and expression of the CK-M isoform, the sperm plasma membrane is also remodelled, thereby promoting the formation of the zona-binding site. This hypothesis was proven by the study of β-1,4,-galactosyltransferase, which is present exclusively in the sperm plasma membrane (Huszar *et al*., 1997). The close correlation between the density of galactosyltransferase on the plasma membrane and both CK activity and the CK-M isoform ratio (both of which originate in the cytoplasmic compartment) in sperm fractions of various states of maturity demonstrated that a simultaneous remodelling process does indeed occur both on the plasma membrane and within the spermatozoa during spermiogenesis. Another aspect of spermiogenetic remodelling relevant from the point of view of sperm function is the relationship between cytoplasmic retention, as evidenced

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by CK activity, other biochemical markers and sperm shape (Rao *et al*., 1989; Huszar and Vigue, 1993; Aitken *et al*., 1994; Huszar *et al*., 1994; Gomez *et al*., 1996; Twigg *et al*., 1998).

The potential relationship between sperm immaturity, as reflected by cytoplasmic retention, and sperm morphology first occurred to us when, following CK immunocytochemistry of human spermatozoa, we found several sperm subpopulations (reflecting the sperm CK activity differences) with different degrees of cytoplasmic retention (Huszar and Vigue, 1993). Normal spermatozoa with clear heads, characteristic of mature spermatozoa that have completed cytoplasmic extrusion, showed the lowest CK content. Other spermatozoa that exhibited patterns of different degrees of CK stippling or even solid CK staining had normal or slightly abnormal morphology. Finally, spermatozoa that displayed extensive cytoplasmic retention were of abnormal morphology and/or amorphous shape. The analysis of the association between CK content and sperm morphology indicated that a relationship exists among increased cytoplasmic retention in the sperm head, a larger head area, increased sperm head roundness, and increased incidence of amorphous sperm heads. We also showed, based on 3000 spermatozoa counted in ten pairs of samples, that the incidence of immature spermatozoa was significantly higher in the supernatant versus the pellet fractions of 40–80% Percoll density gradients (Huszar and Vigue, 1993). Considering that tail sprouting also occurs during spermiogenesis, we postulated that immature spermatozoa which had not completed cytoplasmic extrusion and thus showed arrested spermiogenic development would also have shorter tails.

In the present experiments, our aim was to explore the relationship between sperm biochemical markers and sperm shape, as measured by objective morphometry, in the head, midpiece and tail regions of mature and immature human spermatozoa. This study was facilitated by the recent paper from an Edinburgh group describing an efficient method for the visualization of the midpiece in spermatozoa (Gomez *et al*., 1996). Using the midpiece staining method, which also highlights the contour of spermatozoa, we further studied the relationship between sperm biochemical maturity and sperm morphology. We prepared mature and diminished-maturity sperm fractions and compared the morphometric dimensions of the sperm head, midpiece and tail in order to establish which parameters of the three sperm regions are related or unrelated to sperm maturity.

Materials and methods

Human sperm preparation

Sperm fractions of varying maturities from 20 men (11 normozoospermic and nine oligozoospermic, sperm concentrations: 51.3 ± 7.3 and 11.0 ± 2.0 $\times 10^6$ spermatozoa/ml, respectively) were prepared from liquefied semen by the method, newly developed in our laboratory, of sequential differential centrifugation. For the purposes of this study, we prefer this method to Percoll or other density gradient centrifugation which excludes low density spermatozoa with extensive cytoplasmic retention or swim-up because we can recover ~70% of the spermatozoa, thus providing a more representative sample. The spermatozoa were fractionated based on their density differences: because mature spermatozoa containing only chromatin and plasma membrane are denser than the immature sperm fraction with cytoplasmic retention, mature spermatozoa sediment in the earlier fractions.

The sequential centrifugation is carried out as follows. Liquefied semen is diluted with pre-warmed human tubal fluid medium (Irvine Scientific, Santa Anna, CA, USA)–bovine serum albumin (5 mg/ml) in a 1:2 ratio of seminal fluid:human tubal fluid medium (max: 3.0 ml per 15 ml conical tube). After mixing, the diluted semen is centrifuged at 400 *g* for 4 min. The supernatant is carefully removed and transferred into a second centrifuge tube. The remaining sperm pellet is fraction A. The supernatant of fraction A is centrifuged again at 400 *g* for 4 min. The supernatant of this step is removed and transferred into a third centrifuge tube. The remaining sperm pellet is fraction B. Finally, the third tube is centrifuged at 4000 *g* for 20 min. The pellet of this step is fraction C.

CK activity and CK-M isoform 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 volumes of ice-cold 0.15 M NaCl and 30 mmol/l imidazole (pH 7.0) at 5000 *g* to remove seminal fluid, and the sperm pellets were disrupted by vortexing in 0.1% Triton, 30 mmol/l imidazole (pH 7.0), 10% glycerol, and 5 mM DTT. The homogenate was clarified by centrifugation at 5000 *g*, and aliquots of the sperm extract were subjected to CK activity determinations by a spectrophotometric CK kit (Sigma Co., St Louis, MO, USA).

The isoforms of sperm CK were separated by electrophoresis on precast Agarose gels (Helena Laboratories, Beaumont, TX, USA). The separated CK isoforms were detected by overlaying the gel with a fluorescent CK substrate. The fluorescent bands corresponding to the CK-M and CK-B isoforms were quantified under long-wave ultraviolet light with a scanning fluorometer. The CK-M ratio is expressed as % [CK-M/(CK-M + CK-B)].

Image analysis of the sperm midpiece and tail

These studies were undertaken using the Image-1 analysis system (Universal Imaging Corp., West Chester, PA, USA) in concert with a histochemical stain that targets the sperm midpiece and also highlights the contour of spermatozoa. The modified technique (Gomez *et al*., 1996) utilizes NADH and nitroblue tetrazolium as electron donor and acceptor, respectively, to form a blue-black compound, formazan (Caldwell, 1976). For this procedure, aliquots of A, B and C sperm fractions resuspended to ~20×10^6 spermatozoa/ml concentration, were dried down onto slides, overlaid with 50 µl of nitroblue tetrazolium (3 mmol/l stock in PBS) and 50 µl of NADH (3.5 mM stock in PBS), and incubated for 4 h at 37°C in a humidity chamber. As a consequence of this histochemical procedure, the entire area of the midpiece, including the residual cytoplasm, was stained blue-black (Figure 1a). In each of the A, B and C fractions of the 20 samples (60 fractions in all), 25 cells with well-defined tails were photographed and analysed (75 spermatozoa for each man, and 1509 spermatozoa in the study).

For the morphometry assessment, photographs of individual spermatozoa (10×15 cm, 1000× magnification) were collected onto an IBM PC computer using a Hitachi colour video camera, and the digitized images were evaluated with the Image-1 software system. After delineating the tail, midpiece and head regions (Figure 1b), we traced and determined the dimensions of the tail length and the following parameters in the head and midpiece: diameter, area, long axis, perimeter, and shape factor (roundness: 1.0 is fully rounded). The determinations were carried out in a blinded fashion: the person who carried out the Image-1 analysis did not know the origin of the

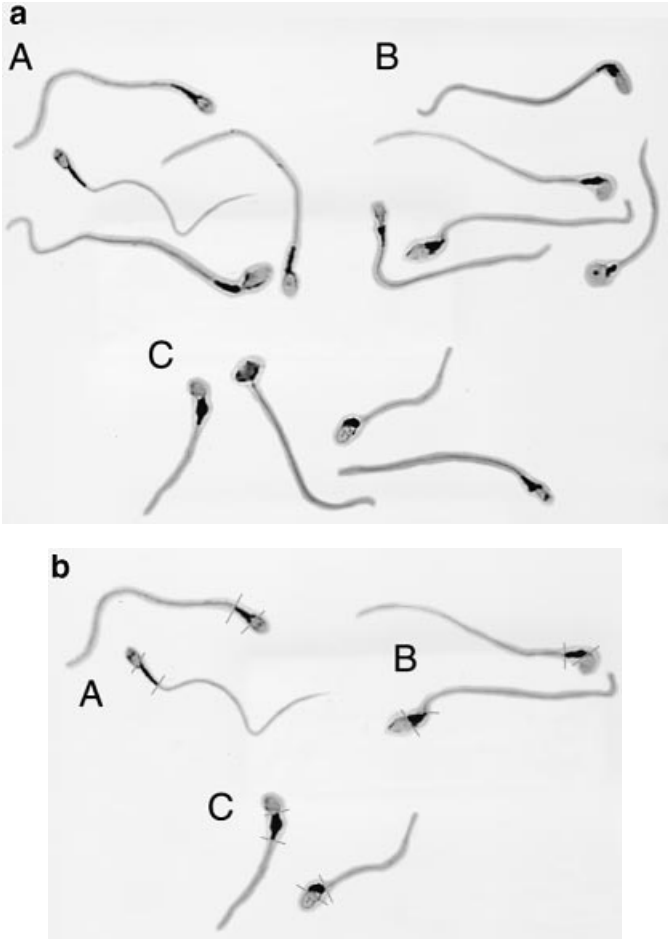

Figure 1. (**a**) Digital images of representative individual spermatozoa arising from the A, B and C fractions, showing recognizable differences in the irregularity of cytoplasmic retention and midpiece abnormalities related to maturity. (**b**) Delineation of sperm originating in the A, B and C fractions into regions of head, midpiece and tail (transverse lines separate regions).

spermatozoa (i.e. fractions A, B or C) in the photographs. Calibration of the system was carried out with photographs of a glass micrometer scale that were taken along with the sperm photographs. Because the actual sizes of the digitized sperm images on the screen were 8.0–12.0 cm long, we could carry out the traces and measurements with high accuracy. Three independent investigators evaluated series of the photographs (~150–200 spermatozoa), with substantially identical results.

Statistical analysis

Data were analysed with SigmaStat software (SPSS Inc., Chicago, IL, USA) on a Micron PC Pentium computer. Differences within and between groups were examined by analysing variance on ranks by means of the Mann–Whitney *U*-test or the unpaired *t*-test, as appropriate. After the analysis of variance on ranks was performed, post-hoc analysis was carried out using Dunn's test to establish the presence of significant differences in the pair-wise comparisons. Data are mean ± SEM. A value of $P < 0.05$ was considered significant.

Results

Sperm recovery and maturity in the A, B and C fractions

We studied the recovery of spermatozoa, CK activity, and CK-M ratios in the 20 semen samples (11 normozoospermic and nine oligozoospermic men, sperm concentrations: 51.3 ± 7.3 and 11.0 ± 2.0 $\times 10^6$ spermatozoa/ml, respectively) subjected to the sequential centrifugation. Among the 11 normozoospermic samples, two showed immature sperm CK-M ratios, while three of the nine oligozoospermic men had sperm CK-M ratios in the mature range. The overall sperm recovery in the normozoospermic samples was 72 ± 3.8%, and the proportions of the recovered spermatozoa in the A, B and C fractions were 70.8 ± 4.6%, 18.7 ± 3.3% and 12.6 ± 1.8%, respectively. The overall recovery in the oligozoospermic group was 64.9 ± 5.6%, and the proportions of recovered spermatozoa in the A, B and C fractions were 59.9 ± 5.9%, 21.0 ± 3.4% and 19.2 ± 3.0% (in both groups A versus B and A versus C, $P < 0.001$). As expected, for the oligozoospermic group (in which six of the nine samples were in the immature CK-M range), the recovery of mature spermatozoa in the A fraction was 16% lower and the recovery of immature spermatozoa in the C fraction was 52% higher than in the respective fractions of the normozoospermic group (in which 10 of the 12 samples were in the mature CK-M range).

In the 20 samples, the declining sperm maturity in the A, B and C fractions, which was due to the increased cytoplasmic retention and lower density of spermatozoa remaining in the supernatant after each centrifugation step, was well reflected by both the CK activities (0.17 ± 0.06, 0.19 ± 0.06 and 0.91 ± 0.3 CK IU/10^8 spermatozoa, A versus C and B versus C, $P < 0.001$) and the CK-M ratios (54.6 ± 5.4%, 38.8 ± 5.6% and 19.8 ± 3.3%, A versus C, $P < 0.001$, A versus B and B versus C, $P < 0.05$) in the three fractions respectively. Thus, using sequential centrifugation, we were able to prepare sperm subpopulations of various maturities from the same specimens.

Sperm dimensions in the A, B and C fractions

In order to examine the relationship between sperm maturity and the sperm morphometric parameters and also to identify the sperm region(s) that differ in mature and immature sperm fractions, we divided the 20 men into two groups based on their sperm maturity in the initial semen. The first group had low CK-M ratios (LCKM: 14.6 ± 7%, $n = 8$, six oligozoospermic and two normozoospermic men). The second group had high CK-M ratios (HCKM: 48.0 ± 4.3%, $n = 12$, two oligozoospermic and 10 normozoospermic men). We examined the spermatozoa originating in the A, B and C fractions (60 fractions in all, examples in Figure 1a and b). From each slide, we took photographs of 25 spermatozoa with fully visible tails ($n = 600$ in the LCKM and $n = 909$ spermatozoa in the HCKM groups).

The data of the LCKM and HCKM groups are presented in Table I. In both groups, the CK-M ratios differed significantly in all comparisons between the A, B and C fractions. The significantly lower CK-M ratios of the respective A versus A (66.8 ± 4.8% versus 36.4 ± 8.1%), B versus B (51.3 ± 6.1% versus 17.4 ± 3.6%) and C versus C fractions (26.3 ± 4.5% versus 10.0 ± 1.7%, $P < 0.001$ in all three comparisons) of the LCKM versus the HCKM groups also confirmed that the overall sperm maturity was higher in the HCKM group than the LCKM group. Regarding the three sperm regions, in the midpiece seven of the 10 morphometric parameters within the

Table I. Morphometry of sperm head, midpiece and tail (mean ± SEM)

	LCKM (n = 8 men, 600 spermatozoa in A+B+C)				HCKM (n = 12 men, 909 spermatozoa in A+B+C)			
	A (n = 201 spermatozoa)	B (n = 211 spermatozoa)	C (n = 188 spermatozoa)	AV	A (n = 322 spermatozoa)	B (n = 320 spermatozoa)	C (n = 267 spermatozoa)	AV
CK-M ratio (%)	36.4 ± 8.1^{ab}	17.4 ± 3.6^{bc}	10 ± 1.7	*	66.8 ± 4.8^{ab}	51.3 ± 6.1^{bc}	26.3 ± 4.5	†
Head								
Diameter (μm)	3.1 ± 0.5	3.0 ± 0.4^{ac}	2.9 ± 0.1	*	3.2 ± 0.1	3.2 ± 0.1	3.3 ± 0.1	NS
Long axis (μm)	5.1 ± 0.1^{ab}	4.9 ± 0.1	5.04 ± 0.1	*	5.3 ± 0.1	5.3 ± 0.1	5.3 ± 0.1	NS
Perimeter (μm)	13.7 ± 0.2	13.3 ± 0.1	13.3 ± 0.2	NS	14.1 ± 0.1	14.2 ± 0.1	14.2 ± 0.1	NS
Area (μm^2)	11.5 ± 0.2	11.1 ± 0.3	11.1 ± 0.3	NS	12.7 ± 0.1	13.0 ± 0.2	13.1 ± 0.3	NS
Shape factor	0.76 ± 0.01	0.77 ± 0.01	0.75 ± 0.01	NS	0.79 ± 0.01	0.79 ± 0.01	1.04 ± 0.24	NS
Midpiece								
Diameter (μm)	1.1 ± 0.1^{ab}	1.6 ± 0.1^{ac}	1.6 ± 0.1	†	1.0 ± 0.1^{ab}	1.6 ± 0.1^{ac}	1.7 ± 0.1	†
Long axis (μm)	6.1 ± 0.1	5.8 ± 0.1	6.1 ± 0.1	*	6.1 ± 0.1	6.1 ± 0.1	5.9 ± 0.1	NS
Perimeter (μm)	14.1 ± 0.2	14.4 ± 0.2^{ac}	14.9 ± 0.2	*	14.0 ± 0.1^{ab}	14.8 ± 0.2^{ac}	14.7 ± 0.2	†
Area (μm^2)	5.7 ± 0.1^{ab}	7.6 ± 0.24^{ac}	7.8 ± 0.3	†	5.2 ± 0.1^{ab}	7.6 ± 0.2^{ac}	8.3 ± 0.3	†
Shape factor	0.35 ± 0.01^{ab}	0.45 ± 0.01^{ac}	0.48 ± 0.03	†	0.33 ± 0.01^{ab}	0.44 ± 0.01^{ac}	0.45 ± 0.01	†
Tail length (μm)	62.5 ± 0.5^{ab}	53.4 ± 0.6^{bc}	49.3 ± 0.7	†	60.1 ± 0.4^{ab}	53.6 ± 0.5^{ac}	51.1 ± 0.6	†

LCKM = lower, HCKM = higher sperm creatine kinase (CK-M) isoform ratios. H = head; MP = midpiece; AV = analysis of variance.
*$P < 0.05$, †$P < 0.001$; ab, ac and bc: significant post-hoc comparisons; NS = not significant.

LCKM and HCKM groups showed differences at the level of $P < 0.001$ and two at the level of $P < 0.05$ among the A, B and C fractions, according to the analysis of variance. In the post-hoc analysis, 15 of the 20 comparisons were significantly different. The midpiece diameter, perimeter, area and shape differed between the A and B and the A and C fractions in both the LCKM and HCKM groups. Similarly, the analysis of variance in the tail length indicated differences at the level of $P < 0.001$ in both the LCKM and HCKM groups. The post-hoc comparisons also indicated differences in line with the CK-M parameters of sperm maturity. The analyses showed no significant differences in eight of the 10 parameters of head dimension, which compared the A, B and C mature and diminished-maturity sperm fractions. In addition, only the head diameter and head long axis in the LCKM group differed between the A and B fractions according to the post-hoc comparison. Thus, according to the morphometric parameters, the various dimensions of the midpiece and tail reflected the differences in sperm biochemical maturity while the head dimensions were unrelated to sperm maturity.

Discussion

In the present work, we studied the correlation between human sperm maturity, as detected by CK activity and CK-M ratios, and objective morphometric parameters in the sperm regions of head, midpiece, and tail. We devised the differential centrifugation method because using this approach the recovery of the spermatozoa depends only on the density and cytoplasmic retention differences, whereas in Percoll or other density gradient centrifugation, the density of the phases preselects the spermatozoa that enter into the gradient, thus excluding very low-density spermatozoa with extensive cytoplasmic retention. The sequential centrifugation method provides three sperm fractions of differing maturity regardless of whether the spermatozoa originate in samples that are in the HCKM or LCKM mature or diminished-maturity range overall, as measured by the CK-M ratios. This further substantiates the fact that each semen sample is composed of sperm fractions of varying maturity. In line with the objective CK measurements, the midpiece parameters and tail length showed significant differences. The head dimensions proximal to the midpiece, however, were similar in the A, B and C mature and diminished-maturity sperm fractions. The increased sperm CK activity reflects the residual cytoplasm in immature spermatozoa, which is also diminished in fertility. We consider the CK-M isoform a developmental marker indicating that spermiogenic remodelling, which occurs simultaneously in the cytoplasmic compartment and on the plasma membrane, has been completed and the spermatozoa are mature. By the time the spermatozoa arrive in the caput epididymis, sperm cellular maturation is completed and neither the CK activity nor the CK-M isoform ratio changes during epididymal descent (Huszar *et al*., 1998).

Three other laboratories have confirmed the relationship between sperm cytoplasmic marker enzymes and sperm function via the study of sperm CK, sperm lactate dehydrogenase (LHDx), and glucose-6-phosphate-dehydrogenase (Casano, 1991; Aitken *et al*., 1994; Orlando *et al*., 1994; Sidhu *et al*., 1998). More specifically, the recent midpiece studies, in accordance with previous data on sperm CK activity and the CK-M isoform ratio, LDH_x activity and LDH_a/LDH_x ratio (Huszar *et al*, 1988a; Huszar and Vigue, 1993; Lalwani *et al*., 1996), showed a close correlation between the residual cytoplasm present in the midpiece and the activities of both the CK and glucose-6-phosphate dehydrogenase enzymes (Gomez *et al*., 1996).

In line with previous complementary studies by the Aitken and Huszar groups that have explored the biochemical markers of sperm function, the present work has one common set of results with Gomez *et al*. (1996) and there are several unique features in the two papers. The common feature is the area of the midpiece measurements in immature and mature sperm fractions, defined by CK-M ratios in our case and by CK and glucose-6-phosphate dehydrogenase activities in the Gomez paper. In spite of the differences in sperm preparation methods

(differential centrifugation which separates spermatozoa strictly based on their density versus Percoll gradients which exclude the low-density/large midpiece sperm fraction) and in midpiece assessment approaches (manual tracing and measurement of individual spermatozoa versus automated area measurement combined with image enhancement), the two studies provided very comparable data. In the Edinburgh study the medians (10th–90th percentile) of midpiece area in the high-density and low-density Percoll populations were 4.7 (3.7–6.3) μm^2 and 5.7 (4.1–8.9) μm^2, which agree very well with our respective values of 5.1 (2.8–6.5) μm^2 and 7.1 (4.3–12.3) μm^2 in the A fraction of HCKM and C fraction of LCKM.

This common point aside, our study differs in scope from that of Gomez *et al*. After establishing that the midpiece area reflects sperm maturity and immaturity, the Gomez paper focuses upon corresponding differences in the rate of lipid peroxidation and sperm function, including sperm motility, acrosome reaction and sperm–oocyte fusion. These data are in full agreement with previous results pertaining to the relationship between cytoplasmic retention, sperm CK-M ratios, zona-binding ability, and the occurrence of IVF pregnancies (Aitken *et al*., 1989a,b, 1994; Huszar and Vigue, 1990; Huszar *et al*., 1992, 1994). Particularly striking is the similarity between the Gomez study's correlation between midpiece area and the reactive oxygen species generation ($r = 0.48$) and the correlation between CK activity and the rate of sperm lipid peroxidation ($r = 0.43$) reported by the Yale group (Huszar and Vigue, 1994). Other findings that support the utility of sperm midpiece measurements are the relationship between midpiece size and rate of lipid peroxidation, the decline in motility in spermatozoa exposed to oxidative stress and the recent data demonstrating that, during spermiogenesis, a remodelling of the sperm plasma membrane occurs, which is likely to promote the formation of the sperm zona-binding sites and ion-channels necessary for the fertilization function (Rao *et al*., 1989; deLamirande and Gagnon, 1992a,b; Huszar *et al*., 1997).

Following a different line of research, in the present study we systematically examined the head, midpiece, and tail regions in sperm fractions of various maturity, as defined by the CK-M ratios, and looked for morphometric features that would reflect sperm maturity. We found that sperm tail length and midpiece dimensions, with the exception of axis length, differed among the mature and diminished maturity sperm fractions, although none of the head dimensions showed corresponding differences. The midpiece long axis length findings contain two points of interest. (i) The axis lengths were not different in the HCKM group, but the analysis of variance indicated an overall difference in the LCKM group without differences between the A, B and C fractions in the post-hoc analyses. The reason for this apparent discrepancy is that the Dunn's post-hoc comparison used is a very conservative test. Other tests indicated a difference ($P < 0.05$) between the A and B groups, but we choose not to mix tests of different levels of rigor in the same Table. (ii) From the physiological perspective one could argue that the length of the midpiece axis should not be subject to substantial changes because it is fixed by the underlying fibre structure, the development of which precedes cytoplasmic extrusion.

Considering the apparent relationship among tail sprouting in the elongated spermatids and the simultaneous events of cytoplasmic extrusion and commencement of CK-M isoform synthesis in the spermiogenesis, we propose a hypothesis incorporating these elements in an integrated scheme of sperm development (Figure 2). In the last phase of spermatogenesis, following the second meiotic division and formation of the round spermatid, the spermiogenic phase of sperm development occurs (Clermont, 1963). During this period, the tail sprouts and the acrosome is formed. As the spermatid elongates further, the cytoplasm that will be left as a residual body in the adluminal area accumulates around the midpiece of the spermatozoa. This process is followed by cytoplasmic extrusion, expression of the CK-M isoform, and the developmental remodelling of the sperm plasma membrane, which facilitates the formation of sites (i.e. zona-binding sites) necessary for sperm–oocyte interaction (Huszar and Vigue, 1990, 1993; Huszar *et al*., 1997). It was somewhat unexpected that all differing morphometric parameters among the A, B and C fractions were in the midpiece and the tail regions of spermatozoa, while head dimensions were similar in the fractions of varying maturity. However, once the data are considered in the light of the related events of spermiogenesis (Figure 2), the connections between sperm immaturity, increased cytoplasmic retention in the midpiece, and shorter tail size become clear. We suggest that the morphological and biochemical parameters of spermatozoa are related because they both reflect the completion of spermiogenic development of mature spermatozoa or the arrest of maturation in diminished-maturity spermatozoa. Further, arrested or delayed spermiogenic development, which results in abnormal morphology due to the retention of the extra cytoplasm (in native or in stained spermatozoa the extra cytoplasm is not perceptible, thus the midpiece–head complex gives the appearence of larger head size), is also reflected in increased CK activity, diminished concentration of the CK-M to CK-B isoforms (lower CK-M ratios), shorter sperm tails and retarded plasma membrane remodelling, which causes diminished zona-binding ability.

In considering the finding that sperm morphometric differences are related to sperm maturity within the midpiece and the tail but not in the head region, it is of interest to review the elements contributing to the assessment of sperm fertility in the strict (Tygerberg) criteria, the sperm morphology system applied most frequently in clinical practice (Kruger *et al*., 1986; Menkveld *et al*., 1990; Grow and Oehninger, 1995). The strict criteria predict diminished fertility in intrauterine insemination and in in-vitro fertilization, elements of which include a larger and elongated sperm head–midpiece complex, a bulging post-acrosomal region, asymmetric insertion of the tail, and tail abnormalities. These sperm features are of common origin with the morphometric parameters of the midpiece and tail which we found to differ significantly in the A, B and C fractions of both the LCKM and HCKM groups. Another sperm evaluation system, the so-called 'multiple anomalies index', is based on multiple features of abnormal morphology in the same spermatozoa (David *et al*., 1975; Jouannet *et al*.,

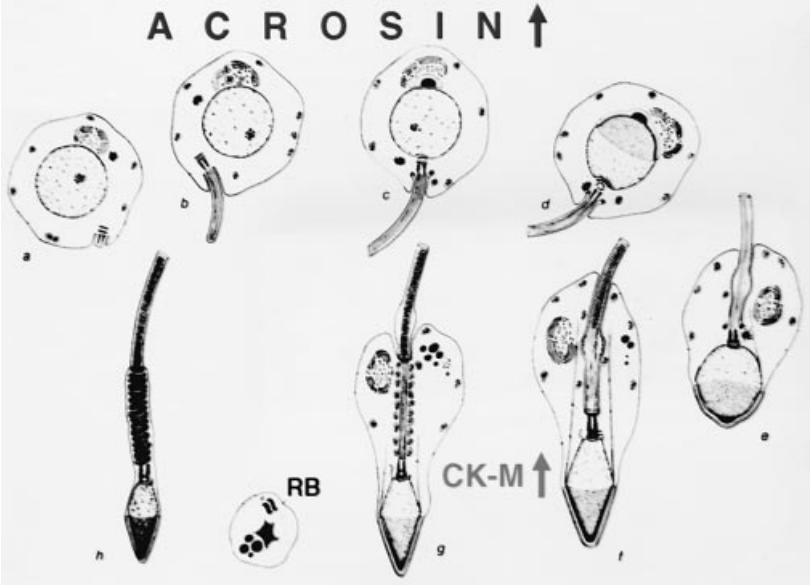

Figure 2. A scheme of human spermiogenesis. Subsequent steps depict an individual spermatid prior to development followed by the stages of the sprouting of the tail, development of the acrosome, expression of the acrosin enzyme (Mendoza *et al*., 1996), elongation of the spermatid, cytoplasmic extrusion, expression of the CK-M isoform and the simultaneous remodelling of the sperm plasma membrane (Huszar and Vigue, 1990; Huszar *et al*., 1997). Conversely sperm immaturity is characterized by shorter tails, cytoplasmic retention, low CK-M isoform ratios and diminished zona pellucida binding (Huszar *et al*., 1994). CK-M = sperm creatine kinase; RB = residual body.

1988). An analysis of the index components (which include an amorphous head–midpiece unit, cytoplasmic retention, and bent and coiled tails) also accords well with the midpiece and tail morphometric features that differ between the LCKM and HCKM groups in our data.

In order to assess the relationship between biochemical markers and sperm morphology, in a blinded study, we carried out simultaneous CK determinations and strict sperm morphology determinations using the Dimensions program (IVOS; Hamilton–Thorne Research, Beverly, MA, USA), which is based on the strict criteria (Menkveld *et al*., 1990; Kruger *et al*., 1996), and the CK parameters developed in our laboratory. In 81 samples, we found close correlations of $r = 0.71$ and $r = -0.74$ between the incidences of abnormal spermatozoa versus CK activity and CK-M ratios, respectively. With respect to clinical utility and the comparative predictive values, the sperm biochemical markers fared better than sperm morphology: in 14 of the 66 normozoospermic semen samples (sperm concentration: $61 \pm 8 \times 10^6$ cells/ml) that did not conform with the correlation, 12 men had abnormal CK activity and CK-M ratio values while only three men showed <15% spermatozoa with normal morphology (Yamada *et al*., 1995).

Based on the data of the present paper, the theoretical question may arise whether sperm morphology or biochemical markers better predict male fertility. This question clearly cannot be decided without rigorous clinical studies, but two points merit consideration. (i) Measurements of CK activity and CK-M isoform ratio are objective, whereas sperm morphology determinations are known to be highly variable among laboratories and technicians. (ii) In accordance with the scheme of Figure 2, a sperm subpopulation which has substantially completed the processes of cytoplasmic extrusion and sperm tail formation during spermiogenesis may experience a late developmental failure that arrests biochemical maturation. Thus, sperm maturity and function in some spermatozoa may be diminished without appreciable changes in sperm morphology.

We can also compare the utility of strict morphology and CK-M isoform ratio in IVF studies, although not on the same population. In an excellent structured review examining the predictive value of strict sperm morphology for IVF outcome (Coetzee *et al*., 1998), 15 articles (of 216 considered) provided sufficient data for statistical analysis. In three key parameters there were significant differences between the groups with <4% and >4% normal forms (fertilization rates: 59.3 versus 77.6%; no transfer rates: 24.0 versus 7.4% and pregnancy rates: 15.2 versus 26.0%). As much as these data may be helpful in the management of patients, they fall short of the optimal goal of diagnostic medicine: to provide inclusion and exclusion criteria (Ombelet *et al*., 1997). The CK-M ratio measurements seem to be more efficacious in predicting IVF failure. In the first blinded study, 22 of the 84 IVF husbands had CK-M ratios in the diminished maturity <10% range and none achieved pregnancy. Of the 22 men, nine were normozoospermic (Huszar *et al*., 1992). A recent retrospective study confirmed these findings. No men with <10% CK-M ratio caused pregnancy, although the proportion of such men was only 15 of 194 couples, because patients with low motile sperm concentrations are now treated with intracytoplasmic sperm injection. For the same reason, however, the representation of normozoospermic men who failed to cause pregnancy has increased (eight of 15) in the <10% CK-M group (Dokras *et al*., 1999).

Due to the ambiguity discussed above between sperm morphology and sperm cellular maturation (Figure 2), we believe that the predictive value of morphology alone cannot be further improved even with 'stricter' criteria. However, morphology coupled with biochemical markers, as is now

attempted in combination with acrosin activity, chromatin structure assessment, and, optimally, an immunoprobe for CK-M, which will allow visualization of individual mature spermatozoa along with the morphology, should be a better approach (Menkveld *et al*., 1996; Duran *et al*., 1998). The data of the present study, identifying the sperm regions that best reflect maturity, will likely further improve the diagnostic utility.

Another aspect of diminished sperm maturity is apparently reflected by differences in chromatin structure, detectable by the DNA-specific fluorescent dye, acridine orange (Evenson *et al*., 1991). It is unclear at present whether the reduced stability of DNA, signified by a shift from green (native, double-stranded DNA) to red (single-stranded DNA), is caused by early spermatogenic faults in DNA replication, defects in the histone–protamine replacement process or by DNA nicks due to the increased rate of lipid peroxidation in immature sperm (Aitken *et al*., 1989a; Huszar and Vigue, 1994; Sakkas *et al*., 1995; Lalwani *et al*., 1996; Steger *et al*., 1998). The value of the sperm chromatin structure assay in predicting male fertility is under investigation in several laboratories. However, a recent epidemiological study in 277 Danish men demonstrated differences with respect to age, smoking habits, and the presence of immature germ cells and leukocytes in the semen (Spano *et al*., 1998).

In the context of sperm maturation, one should also address the distinction between cellular maturation which is a genomically regulated process, and 'epididymal maturation', which involves modification of the spermatozoa in order to improve motility and functional integrity in both resisting premature acrosome reaction and interacting efficiently with the female reproductive tract (Amman *et al*., 1993). We have recently shown in men and in stallions that cellular maturation with respect to cytoplasmic extrusion and the synthesis of the CK-M isoform is completed by the time the spermatozoa arrive in the caput epididymidis. There was a high incidence of mature spermatozoa, with CK values similar to that of ejaculated spermatozoa, in all epididymal segments (Huszar *et al*., 1998). Similarly, in a study of prostatic carcinoma patients, there was only a slight improvement in sperm morphology among sperm fractions arising from the efferent ducts or from the corpus and cauda epididymides, whereas sperm function attributes, such as motility and accrosome response to calcium ionophore showed several-fold increases in the cauda spermatozoa (Yeung *et al*., 1997). Thus, in the light of the functional but not structural sperm changes, the question arises whether the epididymal process should not be called 'epididymal conditioning' rather than 'maturation' in order to distinguish events that are influenced by the local environment of the epididymis.

Several groups have reported specific alterations in sperm properties due to epididymal exposure *in vivo* or *in vitro* (Turner, 1995; Boue *et al*., 1996; Akhondi *et al*., 1997). For instance, recent work demonstrated changes in sperm membrane anisotropy and the content of phosphatidylcholine, which is known to stimulate motility of epididymal sperm *in vitro* (Haidl and Opper, 1997). In view of the spermiogenic remodelling (Huszar *et al*., 1997), one may consider whether these lipid changes are homogeneous in all spermatozoa, or whether there are variations related to sperm cellular maturity and plasma membrane structure in acquiring the optimal set of lipid and protein modulators.

In conclusion, we have established the relationship between sperm morphology and the biochemical markers of sperm maturity based on principles of the cell biology of human spermiogenesis. In addition to a better understanding of this relationship, this work has a future practical application because the morphometric dimensions associated with mature sperm fractions provide objective values that will facilitate the computer-assisted determination of normal sperm morphology in semen samples. This method can be further enhanced by fluorochrome-coupled probes specific to the biochemical markers of sperm maturity, which may enhance the utility of the morphology studies in men with diminished fertility who may show disturbance of synchronicity in the spermiogenic maturation processes.

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