Anna-Eszter Gergely

COMPUTER-BASED ASSESMENT OF THE RELATIONSHIP BETWEEN BIOCHEMICAL AND MORPHOLOGYCAL SPERMATOZOA-PARAMETERS AND SPERM MATURITY

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Albert Szent-Gyorgyi Medical University
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INTRODUCTION

Choosing a mature sperm for use in assisted reproduction techniques such as intracytoplasmic sperm injection (ICSI) - in which a single spermatozoa is injected into an oocyte - is very difficult without an objective signal of sperm maturity. The well-known fact that the sperm maturation process, which can be expressed by morphological and biochemical parameters is highly error-susceptible with regard to its genetic content, is a worldwide concern. Researchers have focused their efforts on finding biochemical, morphological or other functional markers of mature, genetically normal spermatozoa that would be detectable using simple laboratory tests, with non-invasive markers in order to maintain their viability and without contaminating them with potentially harmful reagents.

In various laboratories the development of different approaches were initiated for the evaluation of selected sperm functions beyond the classical semen analysis parameters, which includes sperm concentration, motility and velocity measurements. The assessment of sperm motility is achieved using the swim-up procedure (Makler et al., 1984; Huszar and DeCherney, 1987) and computer-assisted analysis of sperm motion patterns (Katz et al., 1985), while the hypoosmotic swelling procedure probes the sperm membrane integrity (Jeyendran et al., 1984). Other tests such as the sperm stress test (Alvarez et al., 1996), the acrosome integrity and functional tests (Liu and Gordon, 1996) and the zona-free hamster ova penetration assay, which reflects also the ability of sperm to participate in sperm-oolemma fusion (Aitken et al., 1987; Wolfe et al., 1996) are all unreliable in predicting male fertility and particularly infertility, which is more important in couples with unapparent male factor or unexplained infertility (Sukcharoen at al., 1996; Ombelet et al., 1997).

Human spermatozoa are generated by a complex developmental process, during which the immature, elongated spermatid ejects its superfluous cytoplasm, condenses its nucleus and grows a tail. Simultaneously with the spermiogenetic event of cytoplasmic

extrusion the developing sperm undergo biochemical remodeling of their enzymatic apparatus.

It has been established by Huszar et al. during the past years that the sperm creatine-phosphokinase (CK) and the ratio of its two isoenzymes, CK-M (mature muscle-type CK) to the CK-B (immature brain-type CK) are objective biochemical markers of sperm maturity and fertilizing potential.

His research was initially focused on the activity of creatine-phosphokinase enzyme in the spermatozoa, which is a key enzyme in the synthesis and transport of energy, utilized mainly to fuel their locomotion by phosphorylating ATP in the mature sperm tail. Studying the correlation between sperm CK activity and sperm concentrations in normozoospermic and oligozoospermic men, they found significant metabolic differences among the two groups, with increased levels of CK activity in the lower sperm count group (Huszar et al., 1988a).

Furthermore, using the immunocytochemical method of CK-staining of the individual spermatozoa it has been demonstrated that the increased amount of CK in the immature sperm is due to disorders or arrest in the cytoplasmic extrusion process (Huszar and Vigue, 1993).

During spermiogenesis a developmental isoformic switch occurs with regard to the CK isoenzymes; the B isoform, which is a brain-type CK, will be replaced by the M-isoform, which has similar electrophoretic properties with the muscle-type variant (Huszar and Vigue, 1990).

The ratio of the CK-M and CK-B isoforms in sperm specimens with various levels of maturity presented significant differences among mature and immature sperm.

Further studies were aimed at the existence of a close relationship of sperm CK activity and the calculated CK-M ratio [%CK-M/(CK-M+CK-B)] with the sperm fertilizing potential, as demonstrated by the occurrence of pregnancy in couples who underwent "in vitro fertilization" (IVF) procedures (Huszar et al., 1990). In a blinded study of couples treated with IVF for male factor infertility, the sperm concentration and motility characteristics did not show a predictive value with respect to oocyte fertilization or pregnancy outcome, however the sperm biochemical parameters of CK activity and CK-M ratios did (Huszar et al., 1992). The explanation for this predictive value was

highlighted by a study which demonstrated that immature sperm with excess retained cytoplasm and high CK activity were unable to bind to the zona pellucida, the glycoprotein shell that coats the oocyte, because the development of the zona-binding site is part of the spermiogenetic remodeling (Huszar et al., 1994).

In our study, supported by the Fogarty Foundation International Collaborative Award, we examined the utility of objective CK markers in the evaluation of Hungarian men who presented themselves for semen analysis at the Department of Obstetrics and Gynaecology, Albert Szent-Gyorgyi Medical University, Szeged, Hungary. The previous findings with respect to the observation that objective biochemical CK markers provide a better assessment of sperm maturity than the sperm concentrations in the samples was confirmed in the Hungarian population.

Examining the biochemical processes which are essential steps of the spermiogenesis, it has been demonstrated that lower CK activity levels (result of cytoplasmic extrusion) and higher CK-M ratios (expression of the CK isoform switch during maturation) were accompanied by significant cell membrane remodeling. During sperm maturation, the decline in CK activity and development of CK-M isoform occur simultaneously with the decline of the membrane enzyme β-1,4-Galactosyltransferase (GalTase), a key element in sperm-zona pellucida binding in rodents (Huszar et al., 1997).

The close correlation between the expression of GalTase on the sperm surface and both CK activity and CK-M isoform ratio (which are cytoplasmic constituents) in subpopulations of sperm with various maturity emphasized that a simultaneous remodeling process occured on the plasma membrane and within the cytoplasm during spermiogenesis. Considering these results, an overall hypothesis can be formulated: sperm with high retained cytoplasmic and CK content, intense immunostaining and lower CK-M ratios are immature and deficient in various functions, including zona-binding and fertilizing potential (Huszar G, 1994).

The complex entanglement of spermiogenetic events that have as accomplishment the mature sperm with functional integrity and the cytoplasmic extrusion process can be expressed by the relationship between CK activity, other biochemical markers and particularly by the sperm shape, which reflects the retention of cytoplasm in immature

sperm (Rao et al., 1989; Huszar and Vigue, 1993; Huszar et al., 1994; Aitken et al., 1994; Gomez et al., 1996).

As demonstrated during previous studies of sperm subpopulations with various degrees of cytoplasmic retention (Huszar and Vigue, 1993), the CK-immunostaining was positively correlated with amorphous shape and/or abnormal morphology. The cytoplasmic retention as shown by CK activity was associated with a larger sperm head area, increased head roundness and increased incidence of amorphous sperm heads.

In our experiments the aim was to examine the relationship between sperm biochemical markers and sperm shape, as measured by computer-assisted morphometry, in the head, midpiece and tail regions of mature and immature human spermatozoa.

Using the recently described midpiece staining method (Gomez et al., 1996) which visualises both the midpiece and sperm head area, we focused our attention on the relationship among sperm maturity as expressed by biochemical markers and morphometric dimensions of the sperm head, midpiece and tail. In order to determine those parameters of the three sperm regions which are related to sperm maturity, we prepared groups of sperm fractions with different levels of maturity and measured their CK activity and CK-M isoform ratio, coupled with simultaneous computer-based morphometrical assessment of 1509 spermatozoa. The studies were carried out in a blinded fashion in two laboratories; in the first laboratory the biochemical staining and photographing of the sperm occurred, while the computerized morphometrical measurements of individual spermatozoa took place in the second laboratory without knowing in which of the six maturity groups the sperm to be evaluated, have originated.

MATERIALS AND METHODS

Sperm Protein Extraction and CK Measurements

Specimens were initially evaluated for sperm concentration and motility as part of the routine sperm analysis. Samples with concentrations $<20\times10^6$sperm/ml were considered oligozoospermic and those of $>20\times10^6$sperm/ml were considered normozoospermic. For the sperm CK measurements each semen sample was washed with 15 to 20 volumes of ice-cold 0.15 M NaCl, 0.03 M imidazole, pH 7.0, at 5.000×g for 15 minutes to remove all seminal fluid contaminations. The resulting sperm pellet was resuspended in ice-cold 0.03 M imidazole, pH 7.0, 10% glycerol, 5 mM dithiothreitol, 0.1% TritonX-100. The sperm were disrupted by vortexing vigorously for 30 seconds. The sperm extract was clarified by centrifugation at 5.000×g, and aliquots were subjected to CK activity determinations by spectrophotometry using a CK kit (Sigma Chem. Co, St. Louis, MO).

Aliquots of semen were washed with 10-15 volumes of ice-cold 0.15 M NaCl and 30 mM imidazole (pH 7.0) at 5.000xg to remove seminal fluid, and the sperm pellets were disrupted by vortexing in 0.1% Triton, 30 mM imidazole (pH 7.0), 10% glycerol, and 5 mM DTT. The homogenate was clarified by centrifugation at 5.000xg, and aliquots of the sperm extract were subjected to CK activity determinations by a spectrophotometric CK kit (Sigma Co., St. Louis, MO).

Human spermatozoa preparation for morphological assessment

Sperm fractions of varying maturity were prepared from liquefied semen by the newly developed method of sequential differential centrifugation. For the purposes of our morphological assessment study, we preferred this method to Percoll or other density

gradient centrifugation which excludes low density sperm with extensive cytoplasmic retention or swim-up, because using this technique we were able to recover approximately 70% of the sperm, thus providing a more representative sample. During this procedure the sperm were fractionated based on their density differences: mature sperm containing only chromatin (nucleus) and plasma membrane are denser than the immature sperm fraction with cytoplasmic retention, thus mature sperm sedimented 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) -5 mg/mL bovine serum albumin 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 400xg for 4 minutes. 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 400xg for 4 minutes. 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 4.000xg for 20 minutes. The pellet which results from this last centrifugation is *fraction C* (FIGURE 6).

CK-M Isoform Ratio Measurements

The CK-M and CK-B isoforms of sperm CK were separated by electrophoresis on precast Agarose gels (Helena Laboratories, Beaumont, TX). 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 (FIGURE 2). The CK-M ratio is expressed as % [CK-M/(CK-M + CK-B)].

Image Analysis of the Sperm Head, Midpiece and Tail

These studies were undertaken using the Image-1 analysis system in concert with a histochemical stain that targets the sperm midpiece and also highlights the contour of sperm. The technique, as modified and described by 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 in about $20x10^6$ million sperm/mL concentration, were dried down onto slides, overlaid with 50 µl of nitroblue tetrazolium (3 mM stock in PBS) and 50 µl of NADH (3.5 mM stock in PBS), and incubated for 4 hours at $37^{O}C$ 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 7a). 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 analyzed (75 sperm for each man, and 1,509 sperm in the study).

For the morphometry assessment, photographs of individual sperm (10x15 cm, 1000x 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 (Universal Imaging Corp. West Chester, Pennsylvania). After delineating the tail, midpiece and head regions (FIGURE 7b), 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 sperm (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 photos. Because the actual size 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 (about 150-200 sperm), with substantially identical results.

Statistical analysis

Data were analyzed with SigmaStat software (SPSS Inc., Chicago, IL) on a Micron PC Pentium computer.

For the CK studies we used Student t-test and linear regression for data analysis in the study population composed of the normozoospermic and oligozoospermic men who presented for semen analysis at the Andrology laboratory of the Department of Obstetrics and Gynaecology, Albert Szent-Gyorgyi Medical University, Szeged, Hungary, between 1993-1996. The normality of distribution was established by the Kolmogorov-Smirnov test, while abnormally distributed data was evaluated by the Spearman Rank correlation test.

For the morphometry studies differences within and among groups in the study population of the 20 men who presented at the Sperm Physiology Laboratory, Department of Obstetrics and Gynaecology, Yale University School of Medicine, New Haven, Connecticut, 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 the 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 CK Activity in Normozoospermic and Oligozoospermic Samples of Hungarian Men

The Huszar laboratory had previously demonstrated that creatine phosphokinase (CK) activity is a measure of cellular maturity and fertilizing potential in human spermatozoa.

To further assess the objectivity of CK parameters in predicting sperm maturity within semen specimens with various sperm concentrations, we determined in a Hungarian study population the sperm CK activities in 56 normozoospermic (concentration: $54.3\pm3.5\times10^6$sperm/ml) and 53 oligozoospermic (concentration: $7.4\pm0.6\times10^6$sperm/ml) semen samples. After measurements, as expected, the CK activities were six times higher in the oligozospermic than in the normozoospermic group (1.19 ± 0.15 IU/10^8sperm vs. 021 ± 0.02 IU/10^8sperm, $P<0.001$). We found an inverse correlation between CK activity and sperm concentration in the 109 samples ($r=-0.70$, $P<0.001$, $N=109$, FIGURE 3).

In order to study the distribution of high and low CK activity among various sperm concentration groups, we examined the relationship between CK-activity and sperm concentrations by dividing the 109 patients into four groups: severely oligozoospermic men ($<10\times10^6$ sperm/ml, N=40), oligozoospermic men ($10\text{-}20\times10^6$ sperm/ml, N=13), men with low normal sperm concentration ($20\text{-}30\times10^6$ sperm/ml, N=11) and normozoospermic men with sperm concentrations of $>30\times10^6$sperm/ml (N=45). In accordance with the inverse correlation between sperm concentrations and CK activity in the 109 samples, the mean CK activities were highest in the severely oligozoospermic men and lowest in the $>30\times10^6$sperm/ml group (FIGURE 4). There was also an incremental decrease in CK activity among sample groups with increasing sperm concentrations, and the mean CK activities differed significantly among the four groups (<10 vs. 10-20, $P<0.05$; <10 vs. 20-30 and >30, $P<0.001$; 10-20 vs. 20-30 and >30, P<

0.05; 20-30 vs. >30, $P<0.05$). This indicates that there is a higher incidence within the low sperm concentration groups of men showing elevated CK activities due to immature sperm with retained cytoplasm.

Furthermore targeting the study of the relationship between sperm concentrations and sperm maturities in the four patient groups (FIGURE 5), we determined the upper level of normal sperm CK activity as 0.43 CK IU/10^8 sperm, based on the mean+2 SD of the CK activity in the >30 million sperm/ml normozoospermic samples (0.17±0.13 CK IU/10^8sperm). The incidences of samples above and below this value provide information on the proportions of men who are oligozoospermic but have sperm with normal maturity (signifying fertilization potential), or who are normozoospermic but show diminished sperm maturity. As in previous studies, the data indicated a substantial discrepancy between sperm concentration and sperm maturity (Huszar et al., 1988a; Huszar et al., 1988b; Huszar G, 1994). In the severely oligozoospermic and oligozoospermic groups, respectively, 27.5% and 39% of the men had CK values in the normal range. Conversely, in the low normal and normal sperm concentration groups, 38% and 5% of the men had CK values in the abnormal sperm maturity range, indicating unexplained male infertility (immature sperm in normozoospermic man). These data clearly demonstrate that sperm maturity is independent of sperm concentrations in the specimens. The most striking finding was the similarity between the incidences of men with mature sperm in the 10-20 million/ml sperm concentration group and of men with immature sperm in the 20-30 million sperm/ml WHO normozoospermic group, which were 38% and 39%, respectively. Another clinically interesting group is the normozoospermic subgroup of men in the > 30million sperm/ml range with diminished sperm maturity, about 5%.

Computer-assissted morphometry of different biochemical-maturity human spermatozoa

Another major aim of our studies on sperm maturity and function relationship was the examination of the head, midpiece and tail of human spermatozoa, using computerized morphometry, in order to highlight those regions which differ among

mature and diminished maturity spermatozoa. We studied the recovery of sperm, CK activity, and CK-M ratios in the 20 semen samples (11 normozoospermic and 9 oligozoospermic men, sperm concentrations: 51.3±7.3 and 11.0±2.0 $x10^6$ spem/mL, respectively) subjected to the sequential centrifugation. Among the 11 normozoospermic samples, two showed immature sperm CK-M isoform 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 sperm in 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 sperm 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 vs. B and A vs. C, $P<0.001$). As expected, for the oligozoospermic group (in which 6 of the 9 samples were in the immature CK-M range), the recovery of mature sperm in the A fraction was 16% lower and the recovery of the immature sperm 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 sperm 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 sperm, A vs. C and B vs. C, $P< 0.001$) and the CK-M ratios (54.6±5.4%, 38.8±5.6%, and 19.8±3.3%, A vs. C, $P< 0.001$, A vs. B and B vs. 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.

In order to examine the relationship between sperm maturity and the sperm morphometrical parameters and also to identify the sperm region(s) that differ in mature and immature sperm fractions, we divided the 20 men into 2 groups based on their sperm maturity in the initial semen. The first group had low CK-M ratios (LCKM: 14.6±7%, N=8, 6 oligozoospermic and 2 normozoospermic men). The second group had high CK-M ratios (HCKM: 48.0±4.3%, N=12, 2 oligozoospermic and 10 normozoospermic men). We examined the sperm originating in the A, B, and C fractions (60 fractions in all,

examples in FIGURE 7a and 7b). From each slide, we took photos of 25 sperm with fully visible tails (N=600 in the LCKM and N=909 sperm in the HCKM groups).

The data of the LCKM and HCKM groups are presented in TABLE 1. In both groups, the CK-M ratios differed significantly in all comparisons among the A, B, and C fractions. The significantly lower CK-M ratios of the respective A vs. A (66.8±4.8% vs. 36.4±8.1%), B vs. B (51.3±6.1% vs. 17.4±3.6), and C vs. C fractions (26.3±4.5% vs.10.0±1.7%, $P<0.001$ in all three comparisons) of the LCKM vs. 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 7 of the 10 morphometrical parameters within the LCKM and HCKM groups showed differences at the level of $P<0.001$ and 2 at the level of $P<0.05$ among the A, B and C fractions, according to the analysis of variance (AV). 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 8 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 morphometrical 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. This finding is mostly surprising because head shape is the basis of the various sperm morphology systems. It suggests that the head shape (which in sperm without the midpiece staining represents the complex of head and midpiece) and head abnormalities are very much influenced by the contributions of the midpiece.

DISCUSSION

While spermatozoa enter the final stages of differentiation they undergo a remarkable transformation whereby the cytoplasmic component of the cell is removed during the release of the mature sperm from the adluminal area to the seminiferous tubuli. Any residual cytoplasm, which remains unextruded from the immature spermatozoa following spermiogenesis is retained in the midpiece as an irregular cytoplasmic mass surrounding the mitochondrial spiral. Being associated with the presence of high concentrations of cytoplasmic enzymes, the retained extra cytoplasm triggers a set of biochemical interactions. The incomplete maturation provides a possible mechanism for the loss in sperm function which results in diminished fertilizing potential.

The conventional semen analysis, which examines sperm concentration, motility and morphology parameters, failed during previous studies to consistently correlate with pregnancy rates, being insufficient to ascertain the sperm's functional maturity (Huszar et al., 1988b; Sukcharoen et al., 1996). Objectivity in assessing critical sperm characteristics has been the target of several researchers. Methods were developed to highlight specific sperm functions such as the acrosome reaction or the zona-free hamster ova penetration test. The scientific basis of these tests, being the assessment of one particular step involved in the sperm-oocyte interaction, all failed to provide information regarding the sperm's functional integrity as followed by their fertilizing potential. The combination of these functional tests resulted in improved predictive value with regard to male fertility, but the best results were encountered by the hemizona binding assay-sperm motility test association, both of which address essential steps of in vivo or conventional IVF fertilization (Wolfe et al., 1996).

Several studies have been conducted in order to investigate the spermiotic process; their results were all pointing at the correlation among defective sperm function and defects in spermiogenesis that lead to the release of immature spermatozoa from the germinative layer. With the purpose of exploring the clinical and biological significance of these associations and the reliability to differentiate immature from mature spermatozoa, particularly among sperm populations within the same ejaculate, the Huszar

laboratory has focused its attention on the midpiece, a part of the human sperm where complex biochemical processes occur.

The mitochondrion present in the midpiece generates ATP for sperm motility, which is taken to the sperm tail vie the creatin-creatin phosphate shuttle: the ATP is utilized for phosphorylation of creatine to creatine-phosphate, which moves as an energy carrier through the tail. While the sperm movements are consuming ATP, which is hydrolyzed by the tubulin-dynein complex in the flagella, the resulting ADP is continuously rephosphorylated by phosphate transfer and the creatine that lost its phosphate is going to be reconstituted in the mitochondrion (FIGURE 1). The creatine-phosphokinase plays a major role in the control of this energy-shuttle as well as in the tail and at the midpiece level. It can be considered as the key enzyme of the generation, transport and utilization of energy in the human spermatozoa.

In order to asses possible differences in the CK activity of sperm populations with various fertilizing potential, oligozoospermic and normozoospermic men were first compared (Huszar et al., 1988a), based on the assessment that higher infertility rates are associated with lower sperms (particularly with sperm concentration <20 million sperm/ml, although they do not predict fertilization potential). The mean CK activities (expressed in IU/10^8 sperm) in oligozoospermic and normozoospermic specimens were inversely correlated with sperm concentrations, revealing that the semen samples with normal sperm concentration, which are more likely to be fertile, contained sperm with lower CK activities. Examining further the swim-up sperm fraction which presents improved motility, velocity and morphology parameters, the previous observation was sustained by the finding of a significantly lower CK activity in the better quality sperm group. This was particularly evident among oligozoospermic specimens, suggesting that each semen sample is composed of various sperm subpopulations with different biochemical characteristics and different fertilizing potential (Huszar et al., 1988b).

Examining the relationship between sperm concentration, CK activity and fertilizing potential in couples who were treated with intrauterine insemination for male factor infertility, it was clearly delineated that the CK activity parameters and the occurrence of pregnancies highly correlates (Huszar and Vigue, 1990; Huszar et al., 1990). There were significant differences between the initial and swim-up sperm

fractions of the fertile and infertile oligozoospermic group, while the CK values of the fertile normozoospermic group were similar to those determined in the fertile oligozoospermic group. The CK activity value of 0.250 IU/10^8sperm (mean+2SD of normozoospermic men) distinguished between fertile and infertile oligozoospermic specimens (Huszar et al., 1990). Using CK immunocytochemistry and direct visualisation of CK content in individual spermatozoa, the pattern of CK staining varied: in the sperm with normal morphology, just the tail and mitochondrion were stained, while amorphous sperm showed heavy head CK-staining (Huszar and Vigue, 1993).

The study of the relationship between sperm CK concentrations and head morphology was undertaken implementing computer-assisted morphometry in order to compare CK activities with morphologic parameters such as head diameter and the ratio of long vs. transverse axis, which characterizes the regularity or roundness of the head. Irregular head morphology and larger head size was associated with increased CK staining patterns. This led to the recognition that during normal spermiogenesis, as part of this complex biological process, extrusion of the cytoplasm occurred with mature fertile spermatozoa as a final result. Developmental arrest, with incomplete cytoplasmic extrusion will end with the presence of cytoplasmic droplets/residual bodies in immature spermatozoa. It can be concluded that sperm with high concentrations of retained CK and cytoplasmic content, which also exhibit increased size, roundness and other morphologic head abnormalities, have failed to complete cellular maturation with a consequential impairment of various functions including fertilizing potential.

Pursuing further on the biochemical maturity, respectively immaturity in sperm with different fertilizing potential, the CK isoforms in human spermatozoa were examined. CK had two isoforms previously described in the skeletal muscle- the B (brain)-type isoform, and the M (muscle)-type isoform. During the developmental process and after initiation of the contractile activity, the CK-B, which was the isoform almost exclusively present in the fetal life, is continuously replaced by the CK-M isoform, which will predominate in the mature skeletal muscle cell.

Based on this line of evidence, it became interesting to examine whether the switch between these two isoforms at the spermatozoa level would occur during spermiogenetic events (Huszar and Vigue, 1990). The CK-B and CK-M fractions,

separated by agarose gel electrophoresis were used to determine the CK-M isoform ratio [%CK-M/(CK-M+CK-B)].

Concomitantly measuring the CK activities and the CK-M isoform ratios in normozoospermic and oligozoospermic specimens, it was noticed that as in previous studies, the CK values were higher in oligozoospermic specimens.

To examine the possible relationship between these two CK parameters, a linear regression analysis was performed; the correlation between CK activities and CK-M isoforms was found to be highly significant ($r=-0.69$, $p<0.001$). It can be stated that the two CK parameters are related and they can be considered as biochemical markers of the sperm maturity and fertilizing potential.

The role of CK-M isoform ratios in predicting fertilization was highlighted during a blinded IVF study, where the distribution of pregnancies among the oligozoospermic and normozoospermic men was correlated with higher CK-M ratios, namely the pregnancies were caused exclusively by husbands in the CK-M Fertile group. Following the regression line between CK activities and CK-M isoform ratios a 10% CK-M isoform value was identified as the coresponding cut-off value for the 0.250 CK IU/10^8sperm CK activity, thus according to this value the CK-M Fertile men with >10% CK-M ratio and CK-M Infertile men with <10% CK-M ratio were defined (Huszar et al., 1992). Of those 22 men who had CK-M ratios in the diminished maturity <10% range, none achieved pregnancy, regardless of the fact that 9 of them presented normal sperm concentrations.

In our study of the Hungarian men population, we examined sperm CK activity and confirmed the previous findings with respect to CK differences among the groups with various sperm concentrations, which support the observation of the lack of a consistent relationship between sperm concentration and sperm maturity. While an overall inverse correlation was present between sperm concentration and CK activities (FIGURE 3), sperm CK activities showed six times lower values in the normozoospermic group than those encountered in the oligozoospermic specimens, which are more likely to present a higher percentage of immature spermatozoa. Dividing further the study population into four groups based on their sperm concentration, the CK activities were found to differ significantly among these groups (FIGURE 4), and a various distribution of sperm with normal or diminished biochemical maturity within the four groups was

also noticed (FIGURE 5). Accordingly, we found that an important percentage both of those men who were included in the severely oligozoospermic group with sperm concentrations <10 million/ml and within 10-20 million/ml range group presented mature sperm. Diminished maturity sperm among normozoospermic men within the >30 million sperm/ml concentration group was also detected. All these findings emphasize the fact that sperm concentrations are inadequate for defining the men's fertilizing potential. The subpopulation of normozoospermic men with diminished sperm maturity stresses the importance of such biochemical markers as the CK activity in detecting unexplained male infertility, where normozoospermia (according to the World Health Organization this includes men with sperm concentrations >20 million sperm/ml semen) is associated with diminished sperm maturity. Almost identical proportions of mature and diminished maturity sperm were observed in the 10-20 and 20-30 million sperm/ml concentration groups, which is another evidence of the ineffectiveness of sperm concentration measurement for the evaluation of sperm fertilizing potential. Although these two groups are categorized as "oligozoospermic" and "normozoospermic" by the WHO, the differences in maturity - as shown by CK activities - do not sustain the necessity of such completely different treatment modalities during IVF procedures as those applied based solely on the sperm count criteria. From the same point of view we should emphasize the importance of the identification in the oligozoospermic group a subpopulation who presented low CK activities, thus diminished fertilizing potential, although their sperm concentration was in the normal range.

Our work, developed on data represented by a Hungarian men population has confirmed the previous results of the USA studies, describing similarly significant differences in the mean CK activities among oligozoospermic and normozoospermic groups, with an essentially various distribution pattern of mature and diminished biochemical maturity sperm within the four sperm concentration groups. In the Hungarian men population the characteristic dissociation between sperm counts and sperm maturities in the semen specimens was also noticed. This study highlights the importance of objective biochemical sperm CK activity parameters in the management of couples with male factor infertility and more specifically, the assessment of unexplained male infertility. One of the primordial requirements of a complete evaluation in couples

who present in IVF programs for male factor infertility is to identify men with normal or diminished fertilizing potential regardless of their sperm concentrations. Sperm CK measurements can contribute to the more efficient work-up of couples with male factor or unexplained infertility, being objective indicators in making such clinical decisions as to when intrauterine insemination can be a therapeutic option for couples with oligozoospermic men, or when an oligozoospermic patient should be advised to undergo varicocelectomy and how long the waiting period afterwards should be. CK activity measurements can be especially beneficial in the work-up of couples where female factor infertility is misdiagnosed because a concomitant male factor is present, personified by an oligozoospermic men with lower than normal sperm concentrations, but with mature, thus fertile sperm. The line of evidence which sustains the usefulness in defining fertility by the means of objective CK tests can be completed with the consideration of the test's simplicity. This biochemical method, which requires just a CK-assay kit and a spectrophotometer after a classical sperm analysis was effectuated, technically is not too complicated to be applied routinely in the clinical practice.

Another important issue considered by the Huszar laboratory is the relationship between sperm biochemical maturity, as emphasized by diminished CK activity and its functional integrity, expressed as the capacity to bind to the oocyte. To examine whether a spermatozoa with immature CK staining patterns is possibly deficient in oocyte recognition and binding sites, CK immunocytochemistry of human sperm-hemizona complexes were employed (Huszar et al., 1994). Sperm heads with clear, slightly increased and very solid CK-immunostaining patterns (which denote the degree of cytoplasmic retention) were identified as mature, intermediate and immature spermatozoa. Using the human sperm-hemizona binding system, which tests the first step of gamete interaction leading to fertilization, there was an exclusive binding to the bisected human oocyte by sperm exhibiting mature CK-staining pattern. This selection is likely to occur because in sperm with incomplete cellular maturity, thus with higher CK activity, the oocyte binding site is not fully developed, leading to diminished fertilizing potential.

The hypothesis that membrane changes take place during spermiogenesis simultaneously with cytoplasmic extrusion and the development of mature sperm-specific

CK-M, was recently demonstrated by the expression pattern of β-1,4-galactosyltransferase (GalTase), a sperm surface element (Huszar et al., 1997). The concentration or density of GalTase in the sperm membrane is an important determinant of sperm-egg binding, because when this protein is overexpressed, sperm have diminished zona-binding ability. Both the CK activities and CK-M isoform ratios were found to be highly correlated with the sperm surface GalTase levels in sperm fractions of varying maturity prepared by Percoll gradient separation (this method divides sperm in subpopulations according to their maturity, since mature sperm and immature sperm with no cytoplasm- to various amounts of retained cytoplasm, have different densities). The changes in GalTase expression during sperm maturation are consistent with the concept of sperm membrane remodeling during the spermiotic process, in agreement with the deficiency in zona binding in immature spermatozoa.

In light of the apparent disruption of spermatogenesis, leading to the retention of excess residual cytoplasm as the etiology of defective sperm function evidence has been provided from a number of independent studies that confirmed the relationship between sperm cytoplasmic marker enzymes and sperm function via the study of sperm CK, sperm lactate dehydrogenase-X (LHDx) and glucose-6-phosphate dehydrogenase (G-6-PDH) activities (Casano et al., 1991; Aitken et al., 1994; Orlando et al., 1994; Sidhu et al., 1998). While the Huszar laboratory has confirmed the correlation of sperm CK activity and CK-M ratios with sperm maturity, complementary studies on lactate dehydrogenase-X (Casano et al., 1991; Lalvani et al., 1996), which is another cytoplasmic enzyme with a role in the sperm's energy household, have found similarities among the development of these biochemical parameters during spermiogenesis.

Another evidence of impaired sperm function observed in immature sperm subpopulations which possess excess residual cytoplasm and high concentration of cytoplasmic enzymes is their association with enhanced peroxidative damage. The reactive oxygen species (ROS) which includes the superoxide anion (O_2-) and hydrogen peroxide (H_2O_2) readily overwhelm the limited antioxidant defenses of human spermatozoa and initiate a lipid peroxidation cascade (Aitken et al., 1989a, 1989b; deLamirande and Gagnon, 1992a, 1992b). That errors of spermiogenesis can result in the creation of oxidative stress was suggested by the positive association observed between

the CK content and the induction of lipid peroxidation (Huszar and Vigue, 1994). G-6-PDH which is present in the residual cytoplasm of immature sperm, in turn significantly correlates with ROS generation, thus the causative link which exist between the presence of excess residual cytoplasm and the induction of peroxidative damage might be expressed in the highly significant correlation observed between the CK and G-6-PDH activities (Aitken et al., 1994). A relationship between incomplete cytoplasmic extrusion and oxidative stress was also suggested by the observation of Rao et al that peroxidative damage to human spermatozoa is associated with morphological abnormalities of the midpiece, where the excess residual cytoplasm is localized (Rao et al., 1989).

In concert with the previous studies that have examined the biochemical markers of sperm function as indicators of the cellular maturity and completed cytoplasmic extrusion, in our work we followed the correlation among human sperm maturity, as detected by the CK-M ratios, and objective morphometrical parameters in different regions of the spermatozoa. In order to investigate this proposed relationship it is essential that methods be developed to include in our data the immature sperm with extensive cytoplasmic retention, thus with very light densities. These sperm subpopulations during Percoll or other density gradient centrifugation are excluded because the density of the phases preselect the sperm that enter into the gradient. With the sequential centrifugation method used in our experiments, the recovery of the sperm depends only on the density and cytoplasmic retention differences, thus it provided us three sperm fractions with varying degrees of maturity, independent of the overall maturity-range in the original specimen, as expressed by higher or lower CK-M ratio values. This further sustains the previous observation that each individual semen sample is composed of sperm subpopulations with different maturities. Dividing the specimens in the higher CK-M (HCKM) and lower CK-M (LCKM) group based on the CK parameters of the original samples, we wished to determine whether the biochemical and developmental heterogeneity characterized by the differences in objective sperm CK maturity-markers is also reflected in the sperm head, midpiece and tail measurements. The observation that increased sperm CK activity, in addition to diminished expression of CK-M isoform, reflects the immaturity of the spermatozoa with excess retained cytoplasm, correlates with the significant differences in the tail length and midpiece

parameters among mature and diminished maturity sperm fractions, as shown by our work. More specifically, the recent midpiece studies, in concordance with the line of evidence offered by previous data on sperm CK activity and the CK-M isoform ratio, LDHx activity and LDHa/LDHx ratio (Huszar et al, 1988a; Huszar and Vigue, 1993; Lalvani et al., 1996), found a close correlation between the residual cytoplasm present in the midpiece and the activities of both the CK and G-6-PDH cytoplasmic enzymes (Gomez et al., 1996).

Assisted by the midpiece staining method described by Gomez et al., we performed computer-assisted sperm analysis combined with image enhancement for head, midpiece and tail region measurements in immature and mature sperm fractions, defined by CK-M ratios, and looked for morphometrical features that would reflect maturity. While tail length and midpiece dimensions, with the exception of midpiece axis length, showed significant differences between mature and immature sperm fractions, none of the head dimensions were correlated with sperm maturity (TABLE 1). With respect to the midpiece long axis findings, it can be remarked that substantial changes should not occur during spermiogenesis in the length of the midpiece, because it is fixed by the development of a complex fiber structure which is completed before the cytoplasmic extrusion commences.

On the other hand, the axis length were not different in the HCKM group, but the analysis of variance showed an overall difference in the LCKM group without differences among the three fractions (A, B and C) 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 test indicated a difference ($P< 0.05$) between the A and B groups, but we decided not to mix tests of different levels of accuracy in the same table.

Based on the recent data, which demonstrates that as the spermatozoa undergoes the maturation process, concomitantly with the rise in CK-M isoform expression, morphological changes in midpiece and tail dimensions occur, the hypothesis of sperm development, as depicted in FIGURE 8, can be formulated. The spermiogenetic phase, which is the last step in the mature sperm development process, commences after the round spermatid is formed as result of the second meiosis in the germinal layer (Clermont, 1963). During this period, the round head of the spermatid elongates, the

acrosome-area becomes apparent, while on the opposite pole the tail sprouting starts. As the spermatid expands further toward its long axis, the residual cytoplasm accumulates in the adluminal area around the midpiece of the sperm, giving birth to the residual body. This process is followed by cytoplasmic extrusion, expression of the CK-M isoform and a simultaneous plasma membrane remodeling, with the development of zona recognition/binding sites, which facilitates sperm-oocyte interaction (Huszar and Vigue, 1990; Huszar and Vigue, 1993; Huszar et al., 1997).

Our data, which showed significant differences among morphometrical parameters of the sperm midpiece and tail regions in the A, B and C fractions, respectively, but none of the head dimensions differed within the three fractions of varying maturity, can be explained in the light of related events of spermiogenesis. As they both express completion of the spermiogenetic process, the morphological and biochemical parameters are correlated measures of sperm maturity. They reflect the achievement of functional integrity by the mature spermatozoa or the arrest in development in immature/diminished maturity sperm. Arrest or delay in the maturation process, which results in morphologic abnormalities (such as larger/amorphous midpiece or midpiece-head complex, which gives the appearance of larger head size) due to the extra retained cytoplasm, is also expressed by increased CK activities, lower CK-M ratios, shorter tails and membrane remodeling deficiencies, with reduced expression of zona-binding sites.

From another perspective, sperm morphometrical differences constitute elements of the strict (Tygerberg) criteria, which is the sperm morphology system most frequently applied in clinical practice for the assessment of male fertility (Kruger et al., 1986; Menkveld et al., 1990; Grow and Oehninger, 1995). The strict criteria elements include a larger and elongated sperm head-midpiece complex, a protruding post-acrosomal region, asymmetrical insertion of the tail with or without tail abnormalities. These morphological anomalies, which predicted diminished fertility in intrauterine insemination and in conventional IVF procedures, are the mirror-image of the differences within the sperm's midpiece and tail morphometrical parameters of both the HCKM and LCKM group of our three sperm fractions. In order to evaluate sperm morphology anomalies, another sperm analysis- the "Multiple Anomalies Index"- was also developed, which evaluates

the sum of various morphological irregularities in the same sperm, such as amorphous head/midpiece, retained excess cytoplasm, disrupted and coiled tails (Jouannet et al., 1988). The results of our study with respect to the differences among the HCKM and LCKM group in the midpiece and tail dimensional characteristics are in concert with the data offered by the Multiple Anomalies Index components, where in infertile couples due to male factor infertility numerous sperm abnormalities were described.

The complex relationship of sperm morphology among the synthesis of new sperm proteins and changes in constituent protein isoforms that occur simultaneously during spermiogenesis, lead to the question whether morphological or biochemical parameters have better predictive value in deciding which is the mature, thus fertile sperm.

That the CK-M ratio measurements are more accurate in predicting IVF failure than the routinely measured sperm parameters has been previously shown by the blinded study of 84 couples, who were treated with intrauterine insemination for male factor infertility (Huszar et al., 1992). None of the 22 men who did not achieve pregnancy presented >10% CK-M isoform ratios (the cut-off value for normal maturity sperm), although 9 of the 22 men were normozoospermic, thus they had normal conventional sperm characteristics. In a second retrospective study, the results were similar; no men with < 10% CK-M ratio caused pregnancy. There were 15 such men in the study population and 9 of the 15 men with CK-M ratios <10% were normospermic (Dokras et al., 1999).

A further attempt to clarify the utility of morphological versus the biochemical parameters is represented by a blinded study, where simultaneous CK parameters and sperm morphology measurements (based on computer-assisted strict criteria - developed by Menkveld et al., 1990; Kruger et al., 1996) were carried out (Yamada et al., 1995). There was a high correlation between sperm CK parameters and strict morphology (r=0.72). However, in the samples in which the parameters did not correspond, the CK markers reflected better than morhology the clinical picture.

A broad review of the utility of strict sperm criteria in predicting the pregnancy outcome in "in vitro fertilization" procedures (Coetzee et al., 1998) reveals that three key parameters were significantly different within the sperm samples with ≤4% and >4%

normal forms (fertilization rates: 59.3% vs 77.6%, no transfer rates: 24.0% vs 7.4% and pregnancy rates: 15.2% vs 26.0%). As it can be drawn from these data, although the scientific basis of patient management modalities during IVF programs is offered by the sperm morphology criteria, their efficacy is not comparable to the objective biochemical markers.

Nevertheless, based on our results it can be concluded that while the CK activity and CK-M isoform ratio measurements are objective, the morphology determinations - both manual or computer-assisted image analysis - suffer from practical and/or device deficiencies, making them tedious or unreliable because of confounding factors and uncontrolled artefacts (Davis and Katz, 1992).

Although a spermatozoa has achieved the morphological features of the completely mature sperm with acrosome area, extruded cytoplasm and long tail, the arrest of a pre-terminal phase in the developmental process with consequent loss in the sperm function can occur. While they are not manifested as appreciable changes in sperm morphology, by means of biochemical tests the late arrest of sperm biochemical maturation and diminished fertilizing potential can be efficiently recognized.

With respect to the complexity of spermiogenesis, the question still remains whether delicate changes in sperm properties occur in order to improve its functional integrity after the genomically controlled maturation is accomplished. In a recent study it has been proved that the cellular maturation, as expressed by the cytoplasmic extrusion and the synthesis of the CK-M isoform is completed when the sperm reaches the caput epididymidis. More specificallly, in all epididymal segments the values of sperm CK-M ratios were comparable with those found in the ejaculated sperm (Huszar et al., 1998).

While it travels through different epididymal regions, the mature sperm experiences changes in its membrane components in order to improve motility and functional integrity in both resisting premature acrosome reaction and interacting efficiently with the female reproductive tract. These alterations, which can be considered as an "epididymal maturation" process, or more correctly "epididymal conditioning", were recently demonstrated by several studies (Turner, 1995; Boue et al., 1996; Akhondi et al., 1997; Haidl and Opper, 1997). With the knowledge on sperm maturation process offered by previous studies, these recent data regarding the effects of the epididymal

enviroment on sperm membrane raise new questions; whether there are differences between various maturity sperm with regard to the degree of epididymal conditioning on lipid and protein surface signals, or whether these changes are affecting the whole sperm population unanimously. Whether the epididymal environment is powerful enough to overcome man-to-man variations or whether there is a different conditioning which affects even the fully developed spermatozoa is another issue.

These questions represent a new paradigm for human sperm biology that challenges scientists. Their sustained efforts to overcome these limitations may redefine how we perform diagnostic and research sperm analysis which reflects sperm maturity and fertilizing potential in the future.

SUMMARY AND CONCLUSIONS

The data of our work, which comes in context with other studies on sperm maturity and fertilizing potential as expressed by biochemical and/or morphological parameters can be summarized as follows:

In oligozoospermic Hungarian men, a group with a higher incidence of diminished fertility, the sperm CK activity were significantly higher than in normozoospermic specimen.

The elevated CK activity is a consequence of increased CK and cytoplasmic protein contents in sperm, which results from the arrest of cytoplasmic extrusion during the final steps in sperm development. Accordingly, sperm with high CK content are immature, featuring different morphological abnormalities and deficiencies in various functions, including zona-binding and fertilizing potential.

In the human spermatozoa there are two isoforms of the CK: CK-M and CK-B. While the B isoform prevails in the immature sperm with incomplete cytoplasmic extrusion, in the mature sperm mostly the M type occurs. Accordingly, the ratio of the CK-M and CK-B isoforms in sperm specimens, being correlated also with the CK activity, is a measure of sperm maturity and functional integrity. The predictive value of CK-M isoform ratios with respect to sperm fertilizing potential was demonstrated in a blinded study of IVF couples, where the rate of pregnancy occurrence or oocyte fertilization, respectively, was highly correlated with the sperm CK-M ratio, independently from the sperm concentration in the samples. Furthermore, it highlights the fact that each semen specimen is the totality of sperm fractions with various maturities.

In addition to the fact that the immature spermatozoa presents increased CK content and lacks expression of the CK-M isoform due to the extra retained cytoplasm, a diminished sperm-oocyte interaction as a result of the lower density of zona-binding sites was also noticed. Assisted by CK immunocytochemistry, the study of sperm-hemizona complexes showed that a preferential binding of mature sperm to the oocyte exists. Also there was a close correlation among the developmental expression patterns of GalTase (a key element of sperm-oocyte binding), the simultaneous decline of sperm CK activity

due to cytoplasmic extrusion and the commencement of CK-M isoform synthesis during spermiogenesis.

These observations are consistent with the concept of simultaneous sperm cytoplasmic extrusion and plasma membrane remodeling during sperm maturation, in concert with the deficiency in zona binding, the more labile membrane lipid structure and the higher CK activity in immature spermatozoa.

The relationship between high CK concentrations, the presence of unextruded cytoplasm and the increased incidence of amorphous sperm features are also reflected by the CK parameters and strict sperm morphology criteria. Conversely, the spermiogenetic events, morphological and biochemical parameters are related, because they both measure sperm maturity or the arrest of maturation in mature and diminished-maturity spermatozoa.

The morphometrical parameters of the sperm midpiece and tail which we observed to be significantly different among various maturity sperm fractions as expressed by the CK-M isoform ratios, found their analogy in the head-midpiece complex anomalies, tail insertion and dimensional defects, which are all elements of the strict morphology criteria.

In seeking to understand the physiological basis of the morphological and biochemical changes which characterize the sperm maturation process, a sperm development scheme was formulated. In the seminiferous tubules of the testes each round spermatid, which commences spermiogenesis after the second meiotic division was completed, ejects all but a small amount of its cytoplasm, develops the acrosome, compacts its nucleus and sprouts a tail. The tiny cytoplasm that is left accumulates around the midpiece and will provide the fuel for sperm motility and functional integrity by means of complex biochemical processes, which are all under enzymatic control. During maturation these enzymes undergo changes with respect to the biosynthesis of their isoforms in order to improve motility and functional integrity so that the mature type isoform (CK-M) is expressed predominantly when the sperm arrives in the caput epididymidis. Simultaneous changes are present on the young spermatozoa's plasma

membrane both in order to display molecules that interact with the zona pellucida and to resist premature acrosome reaction.

Based on principles of cell biology of human spermatozoa and events of spermiogenesis the close relationship between sperm morphology and the biochemical markers of sperm maturity has been established. In addition to the fact that it contributes to the clarification of this relationship, our work has future practical applications. Identifying the sperm morphometrical parameters that best reflect maturity in various sperm specimens, these data can be implemented in computer-assisted sperm analysis systems, enhancing the objectivity and accuracy of such measurements. In order to yield more reliable results, the morphological parameters can be combined with biochemical measurements, based on simple marker-specific fluorochrome-coupled probes. There are already in experimental phase the combinations of morphology with acrosin activity and/or DNA analysis, respectively (Mendoza et al., 1996; Menkveld et al., 1996; Duran et al., 1998; Spano et al., 1998), but even more optimal would be the association of morphology parameters with CK immunoprobe. This will become particularly beneficial in diagnostic medicine by enhancing the utility of morphology studies in men with previously unexplained diminished fertility, which can result from disturbances in spermiogenesis and the sum of the syncronized elements of the maturation process.

REFERENCES

1. Aitken, R.J., Thatcher, S., Glasier, A.F., et al. (1987) Relative ability of modified versions of the hamster oocyte penetration test, incorporating hyperosmotic medium or the ionophore A23187, to predict IVF outcome. *Hum. Reprod.*, **2**,227-231.
2. Aitken, R.J., Clarkson, J.S., Fishel, S. (1989a) Generation of reactive oxygen species, lipid peroxidation and human sperm function. *Biol. Reprod.*, **40**,183-197.
3. Aitken, R.J., Clarkson, J.S., Hargreave, T.B. et al. (1989b) Analysis of the relationship between defective sperm function and the generation of reactive oxygen species in cases of oligoozospermic men. *J. Androl.*, **10**,214-220.
4. Aitken, R.J., Krausz, C. and Buckingham, D. (1994) Relationship between biochemical markers for residual sperm cytoplasm, reactive oxygen species generation, and the presence of leukocytes and precursor germ cells in human sperm suspensions. *Mol. Reprod. Develop.*, **39,** 268-279.
5. Alvarez, J.G., Minaretzis, D, Barrett, C.B., Mortola, J.F., Thompson, I.E. (1996) The sperm stress test: A novel test that predicts pregnancy in assisted reproduction technologies. *Fertil. Steril.*, **65**(2), 400-405.
6. Akhondi, MA., Chaple, C., Moore, HDM. (1997) Prolonged survival of human spermatozoa when co-incubated with epididymal cultures. *Hum. Reprod.*, **12**: 514-522.
7. Boue, F., Blais, J., Sullivan R. (1996) Surface localization of P34H. an epididymal protein, during maturation, capacitation and acrosome reaction of human spermatozoa. *Biol. Reprod.*, **54,**1009-1017.
8. Casano, R., Orlando, C., Serio, M., Forti, G. (1991) LDH and LDHx activity in sperm from normospermic and oligospermic men. *Int. J. Androl.*, **14**, 257-263.
9. Coetzee, K., Kruger, T., Lombard, C., J. (1998) Predictive value of normal sperm morphology: a structured literature review. *Human Reproduction Update*,**4**., 257-263.
10. Clermont, Y. (1963) The cycle of the seminiferous epithelium in man. *Am. J. Anat.*, **112**, 35-51.
11. Davis, R.O., Katz, D.F. (1992) Standardization and comparability of CASA instruments. *J. Androl.*, **13**(1), 81-86.

12. deLamirande, E. and Gagnon, C. (1992a) Reactive oxigen species and human spermatozoa. I. Effects on the motility of intact spematozoa and on sperm axonemes., *J. Androl.* **13,** 368-378.
13. deLamirande, E. and Gagnon, C. (1992b) Reactive oxygen species and human spermatozoa. II. Depletion of adenosine-triphosphate (ATP) plays a role in the inhibition of sperm motility., *J. Androl.* **13,** 379-386.
14. Dokras, A., Habana, H., Giraldo, G. et al. (1999) Sperm cellular maturity and the treatment choice of IVF or ICSI: the contributions of the sperm creatine kinase M-isoform ratio. *Abstract, American Society for Reproductive Medicine* 55th Meeting, Toronto, Ontario.
15. Duran, E.H., Gurgan, T., Gunalp, S., et al. (1998) A logistic regression model including DNA status of spermatozoa for prediction of fertilization in vitro. *Hum. Reprod.* **13**,1235-1239.
16. Gomez, E., Buckingham, D.W., Brindle, J. et al. (1996) Development of an Image-Analysis system to monitor the retention of residual cytoplasm by human spermatozoa - correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *J. Androl.,* **17,** 276-287.
17. Grow, D., Oehninger, S. (1995) Strict criteria for the evaluation of human sperm morphology and its impact on assisted reproduction., *Andrologia,* **27(6),** 325-333.
18. Haidl, G., and Opper, C., (1997) Changes in lipids and membrane anisotropy in human spermatozoa during epididymal maturation. *Hum. Reprod.*, **12**,2720-2723.
19. Huszar, G., Decherney, A. (1987) The role of intrauterine insemination in the treatment of infertile couples: The Yale experience. *Semin. Reprod. Endocrinol.*, **51**, 11-21.
20. Huszar, G., Corrales, M. and Vigue, L. (1988a) Correlation between sperm creatine phosphokinase activity and sperm concentrations in normospermic and oligospermic men. *Gamete Research* **19,** 67-75.
21. Huszar, G., Vigue, L. and Corrales, M. (1988b) Sperm creatine phosphokinase activity as a measure of sperm quality in normospermic, variablespermic and oligospermic men. *Biol. Reprod.*, **38,** 1061-1066.

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

23. Huszar, G., Vigue, L., Corrales, M. (1990) Sperm creatine kinase activity in fertile and infertile oligozospermic men. *J. Androl.* **11**, 40-46.

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

25. Huszar, G. and Vigue, L. (1993) Incomplete development of human spermatozoa is associated with increased creatine phosphokinase concentrations and abnormal head morphology. *Mol. Reprod. Develop.*, **34,** 292-298.

26. Huszar, G. (1994) The role of sperm creatine kinase in the assessment of male fertility. *Reprod. Med. Rev.*, **3**, 179-187.

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

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

29. Huszar, G., Sbracia, M., Vigue, L. et al. (1997) Sperm plasma membrane remodeling during spermiogenetic maturation in men: Relationship among plasma membrane beta-1,4-galactosyl-transferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. *Biol. Reprod.*, **56,** 1020-1024.

30. Huszar, G., Patrizio, P., Vigue L. et al. (1998) Cytoplasmic extrusion and the switch from creatine kinase B to M isoform are completed by the commencement of epididymal transport in human and stallion spermatozoa. *J. of Andrology*, **19,** 11-20.

31. Jeyendran, R.S., van der Ven, H.H., Perez-Pelaez, M., Crabo, B.G., Zanenveld, L.D.J. (1984) Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. *Reprod. Fertil.*, **70**, 219-228.

32. Jouannet, P., Ducot, B., Feneux, D. et al. (1988) Male factors and the likelihood of pregnancy in infertile couples. I. Study of sperm characteristics. *Intl. J Androl.*, **11,** 379-394.

33. Katz, D.F., David, R.O., Delandmeter, B.A., Overstreet, J.W. (1985) Real-time analysis of sperm motion using automatic video image digitization. *Comput. Math. Prog. Biomed.*, **21,** 173-182.

34. Kruger, T.F., Lacqut, F.A., Ozgur, K. (1996) A prospective study on the predictive value of normal sperm morphology as evaluated by computer (IVOS). *Fertil. Steril.*, **66**, 285-291.

35. Kruger, T.F., Menkveld, R., Stander, F.S.H. et al. (1986) Sperm morphologic features as a prognostic factor in in vitro fertilization. *Fertil. Steril.,* **46,** 1118-1125.

36. Lalwani, S., Sayme, N., Vigue, L. et al. (1996) Biochemical markers of early and late spematogenesis: Relationship between the lactate dehydrogenase-X and creatine kinase-M isoform concentrations in human spermatozoa. *Mol. Repr. Dev.* **43:** 495-502.

37. Liu, D.Y.L., Gordon, B.H.W. (1996) Relationship between the zona pellucida (ZP) and ionophore A23187-induced acrosome reaction and the ability of sperm to penetrate the ZP in men with normal sperm-ZP binding. *Fertil. Steril.*, **66**(2), 312-315.

38. Makler, A., Huszar, G., DeCherney, A.H. (1984) Improved techniques for collecting motile spermatozoa from human semen. I. A self-migratory method. *Int J. Androl.*, **7,** 61-70.

39. Mendoza, C., Benkhalifa, M., Cohen-Bacrie, P., et al. (1996) Combined use of proacrosin immunocytochemistry and autosomal DNA in situ hybridisation for evaluation of human ejaculated germ cells. *Zygote*, 4, 279-283.

40. Menkveld, R., Stander, F.S.H., Kotze, T.J.W. et al. (1990) The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Hum. Reprod.*, **5,** 586-593.

41. Menkveld, R., Rhemrev, J.P.T., Franken, D.R. et al. (1996) Acrosomal morphology as a novel criterion for male fertility diagnosis: relation with acrosin activity, morphology (strict criteria), and fertilization in vitro. *Fertil Steril.*, **6,** 637-644.

42. Ombelet, W., Bosmans, E., Janssen, M. et al. (1997) Semen parameters in a fertile versus subfertile population: a need for change in the interpretation of semen testing. *Hum. Reprod.* **12**, 987-993.

43. Orlando, C., Krausz, C., Forti, G. et al. (1994) Simultaneous measurement of sperm LDH, LDHx, CPK activities and ATP content in normospermic and oligospermic men. *Int. J. Androl.* **17,** 13-18.

44. Rao, B., Soufir, J.C., Martin, M. et al. (1989) Lipid peroxidation in human spermatozoa as related to midpiece abnormalities and motility. *Gamete Res.* **24**:127-134.

45. Sidhu, R.S., Sharma, R.K., and Agarwal, A. (1998) Relationship between creatine kinase activity and semen characteristics in subfertile men. *Intern. J. Fertil.*, **43,** 192-197.

46. Spano, M., Kolstad, A.H., Larsen, S.B. et al (1998) The applicability of the flow cytometric sperm chromatin structure assay in epidemiological studies. *Hum. Reprod.* **13**: 2495-2505.

47. Sukcharoen, N., Keith, J., Irvine, D.S., Aitken, R.J. (1996) Predicvtion of the in-vitro fertilization (IVF) potential of human spermatozoa using sperm function tests: the effect of the delay between testing and IVF. *Hum. Reprod.*, **11**(5), 1030-1034.

48. Turner, TT. (1995) On the epididymis and its role in the development of the fertile ejaculate. *J. Androl.*, **16**, 292-298.

49. Yamada, Y., Vigue, L. and Huszar, G. (1995) Sperm creatine kinase parameters and strict sperm morphology in men: Relationship between the biochemical and morphological measures of sperm maturity and fertilizing potential. *American Society for Reproductive Medicine 51st Annual Meeting*, Seattle, Washington.

50. World Health Organization. (1992) *WHO Semen Manual Examination of Human Semen and Cervival Mucus Interaction.* 3rd ed. Cambridge, UK, Cambridge University Press.

51. Wolfe, J.F., Bulwa, S., Ducot, B., Rodriguez, D., Jouannet, P. (1996) Fertilizing ability of sperm with unexplained in vitro fertilization failures, as assessed by the zona-free hamster egg penetration assay: its prognostic value for sperm-oolemma interaction. *Fertil. Steril.*, **65**(6), 1196-1201.

LEGENDS TO FIGURES

FIGURE 1: The sperm creatine-phosphate shuttle model. The sperm mitochondrion and tail are the sites of the ATP production and consumption. The creatine/creatine-phosphate system and the mitochondrial and muscle type CK isoenzymes facilitate the energy transfer between the two compartments.

FIGURE 2: Separation of CK isoforms. A) muscle extract; B) sperm CK isoforms of a normozoospermic/fertile man; C) sperm CK isoforms of an oligozoospermic/ infertile man. (M: CK-M, MB: CK-MB, B: CK-B isoform, respectively)

FIGURE 3: Correlation between CK activity and sperm concentration in the 109 samples. The data were subjected to logarithmic transformation.

FIGURE 4: Distribution of CK activities in the four sperm concentration groups.

FIGURE 5: Proportions of samples with mature and immature spermatozoa in the four sperm concentration groups.

FIGURE 6: The flowchart of the sperm head, midpiece and tail morphometrical study.

FIGURES 7a and 7b:

(**7a**) Representative individual sperm arising from the A, B, and C fractions, showing recognizable differences in the irregularity of cytoplasmic retention and midpiece abnormalities related to maturity.

(**7b**) Delineation of sperm originating in the A, B, and C fractions into regions of head, midpiece, and tail (red lines).

TABLE 1: Morphometry of sperm head, midpiece and tail.

FIGURE 8: A scheme of human spermiogenesis. Subsequent steps depict 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 remodeling 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).

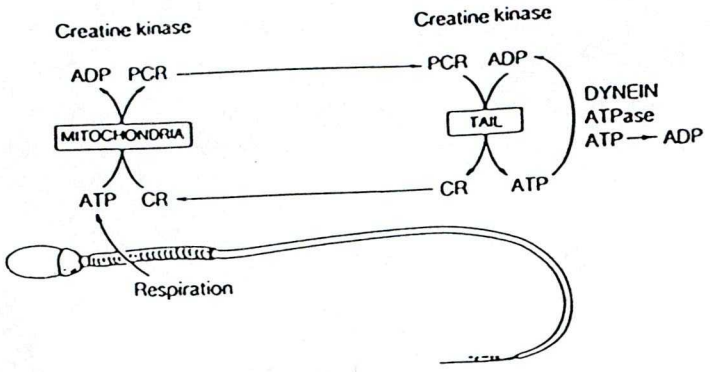

FIGURE 1.

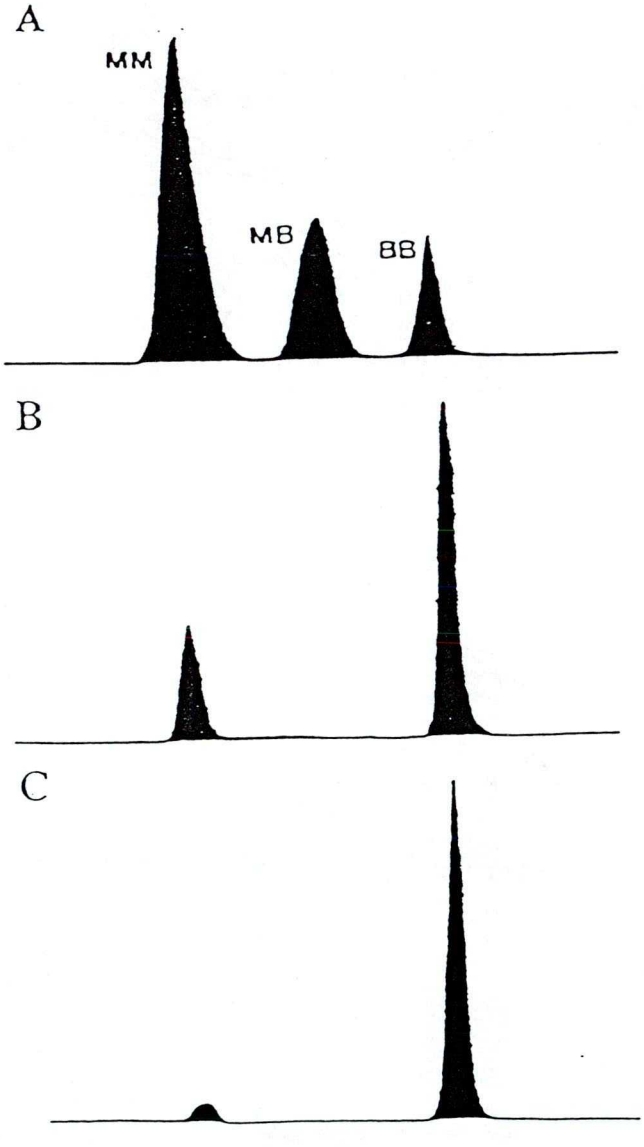

FIGURE 2.

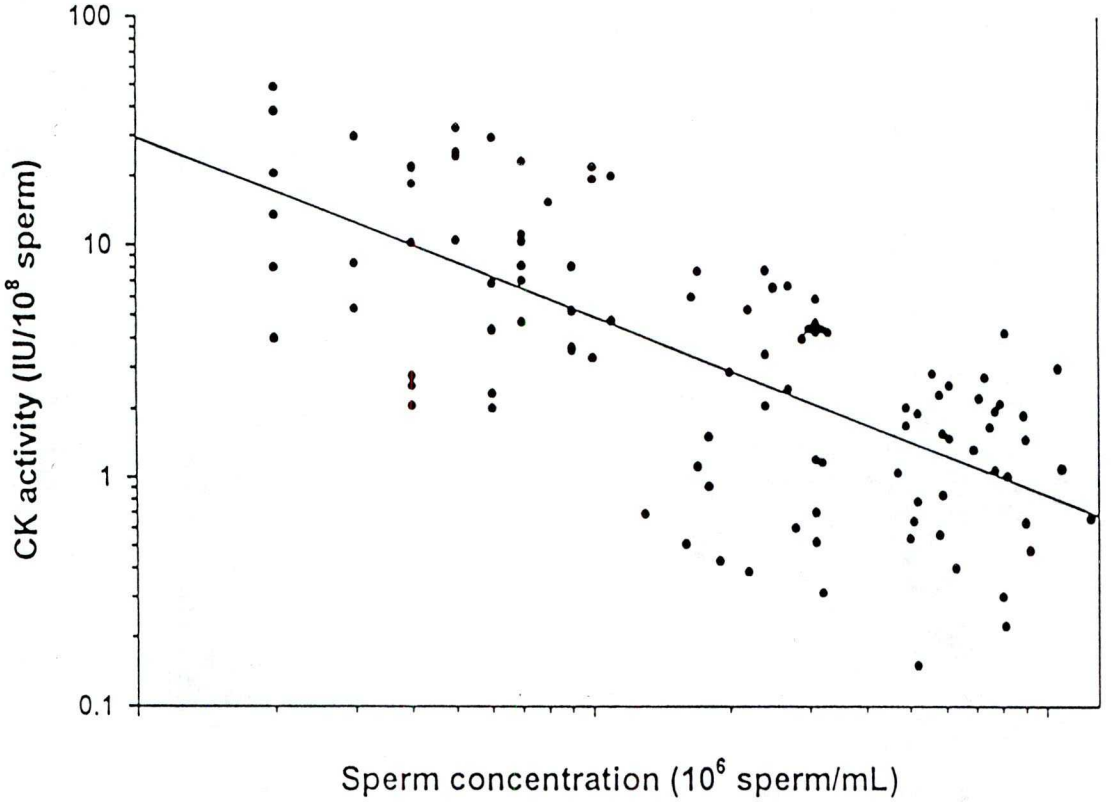
100
10
1
0.1
CK activity (IU/10^8 sperm)
Sperm concentration (10^6 sperm/mL)

FIGURE 3.

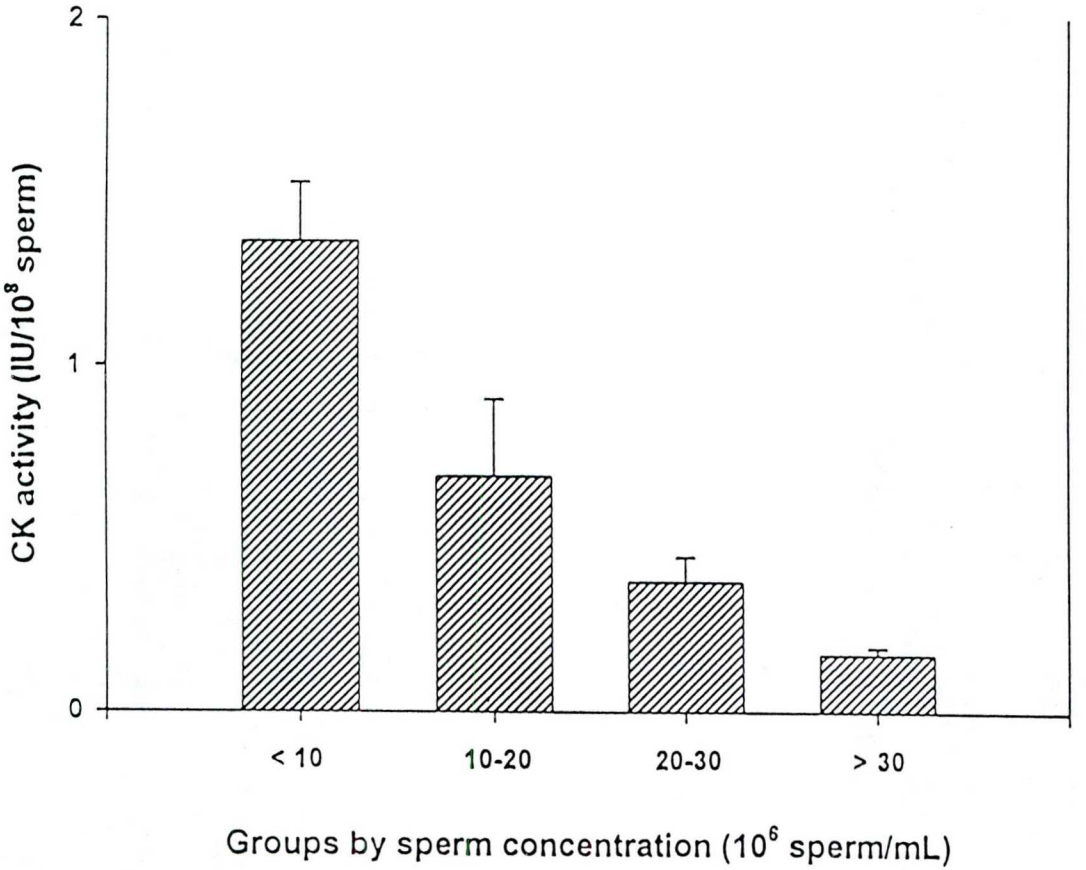
2
1
0
CK activity (IU/10^8 sperm)
< 10
10-20
20-30
> 30
Groups by sperm concentration (10^6 sperm/mL)

FIGURE 4.

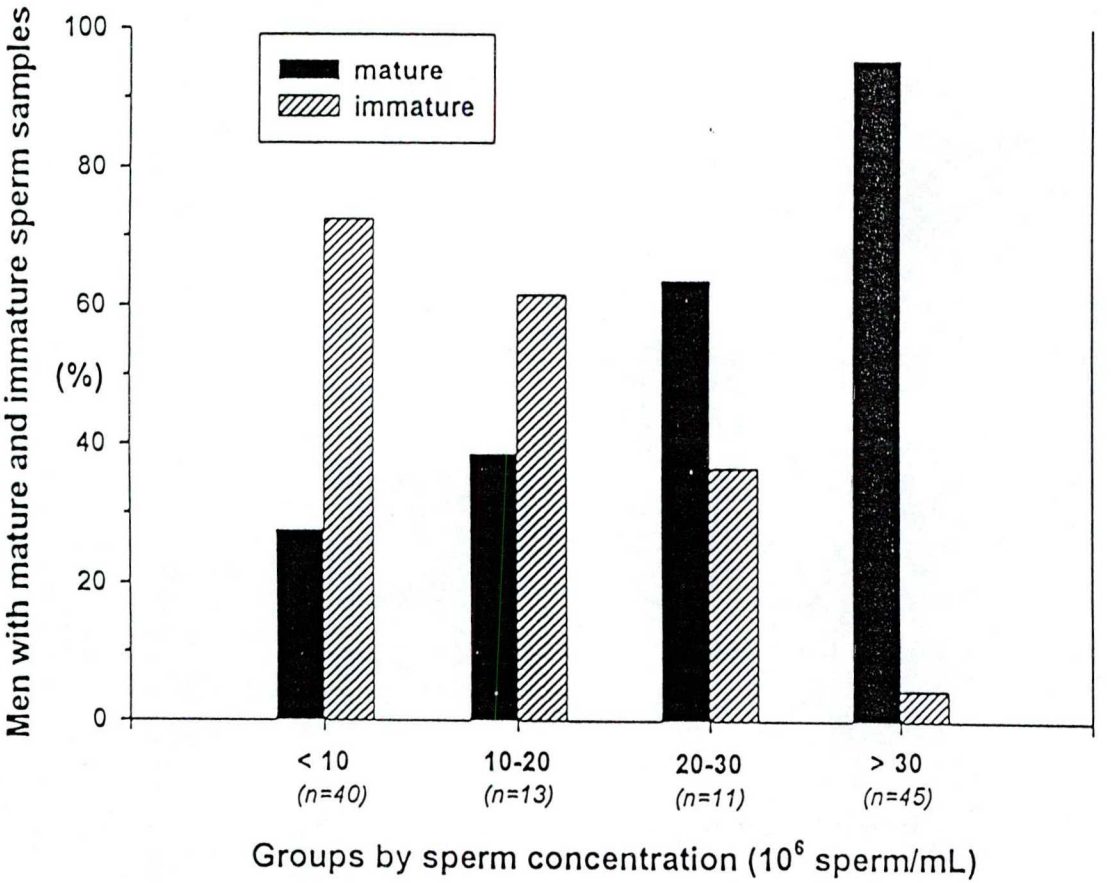
Men with mature and immature sperm samples
100
80
60
(%)
40
20
0
mature
immature
< 10
(n=40)
10-20
(n=13)
20-30
(n=11)
> 30
(n=45)
Groups by sperm concentration (10^6 sperm/mL)

FIGURE 5.

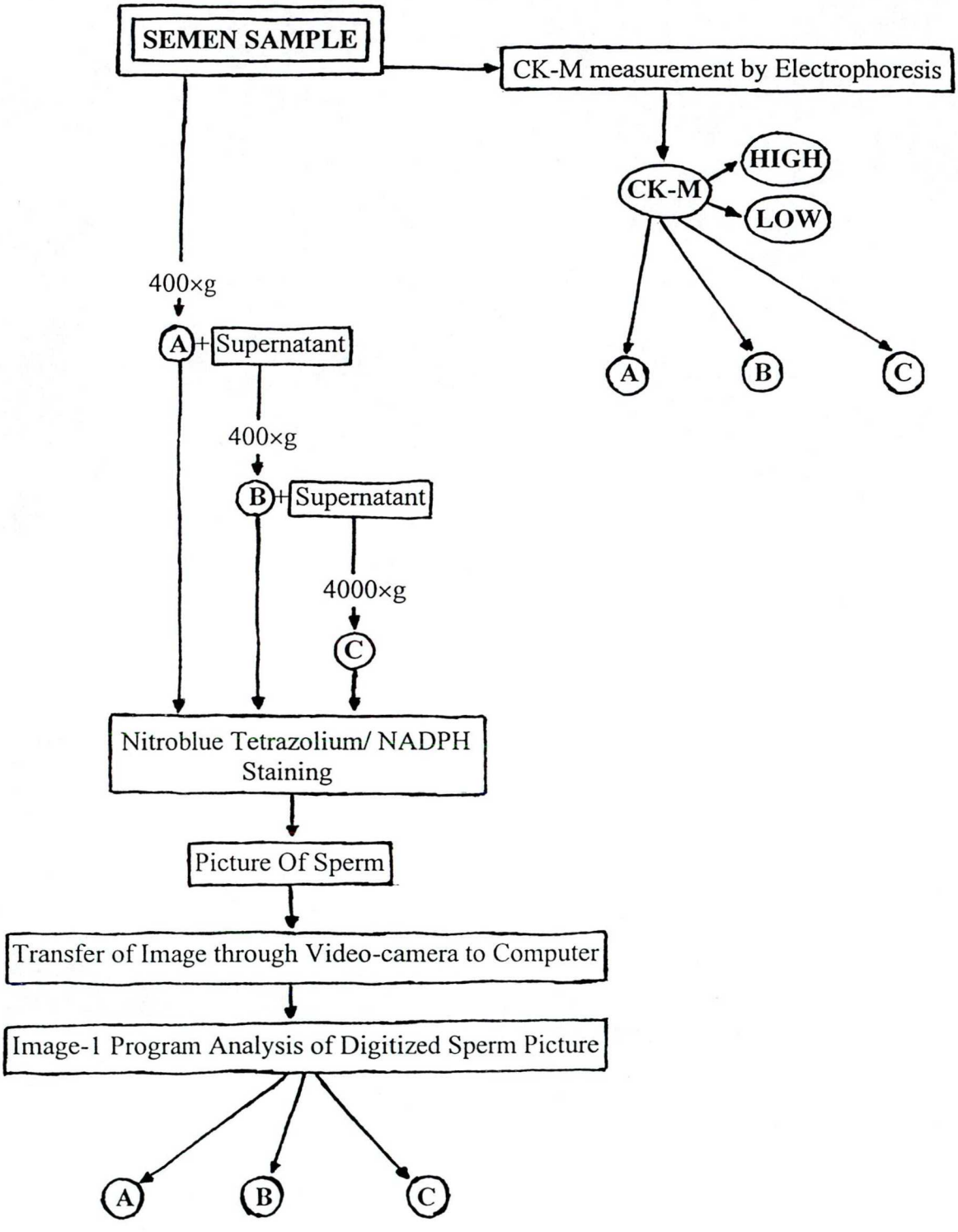

FIGURE 6.

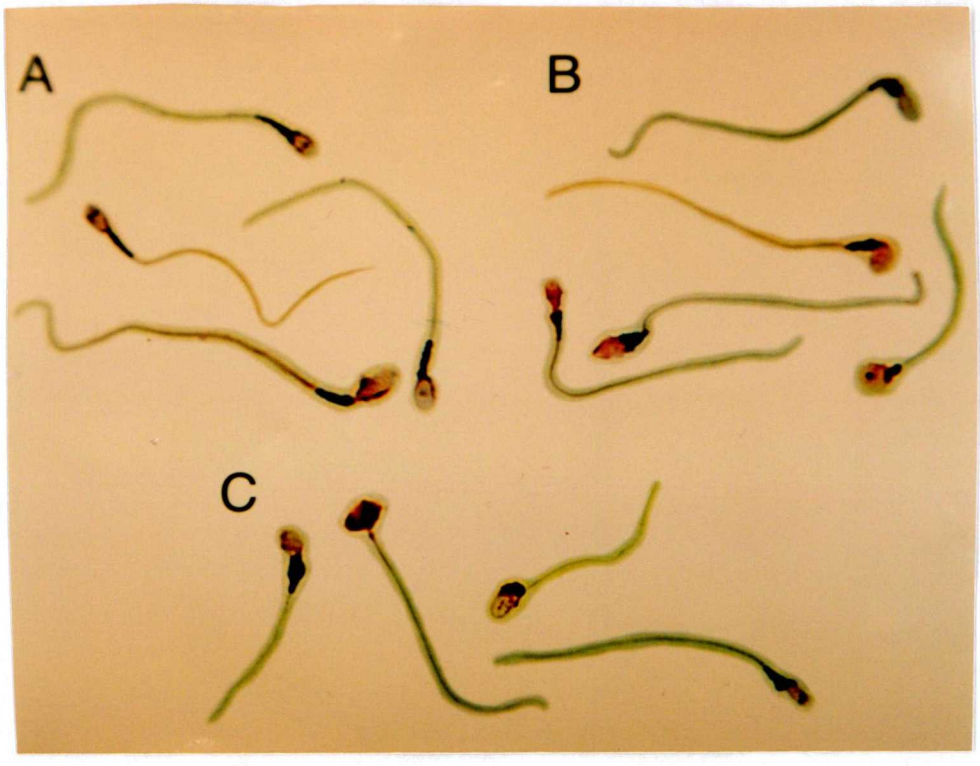

FIGURE 7a.

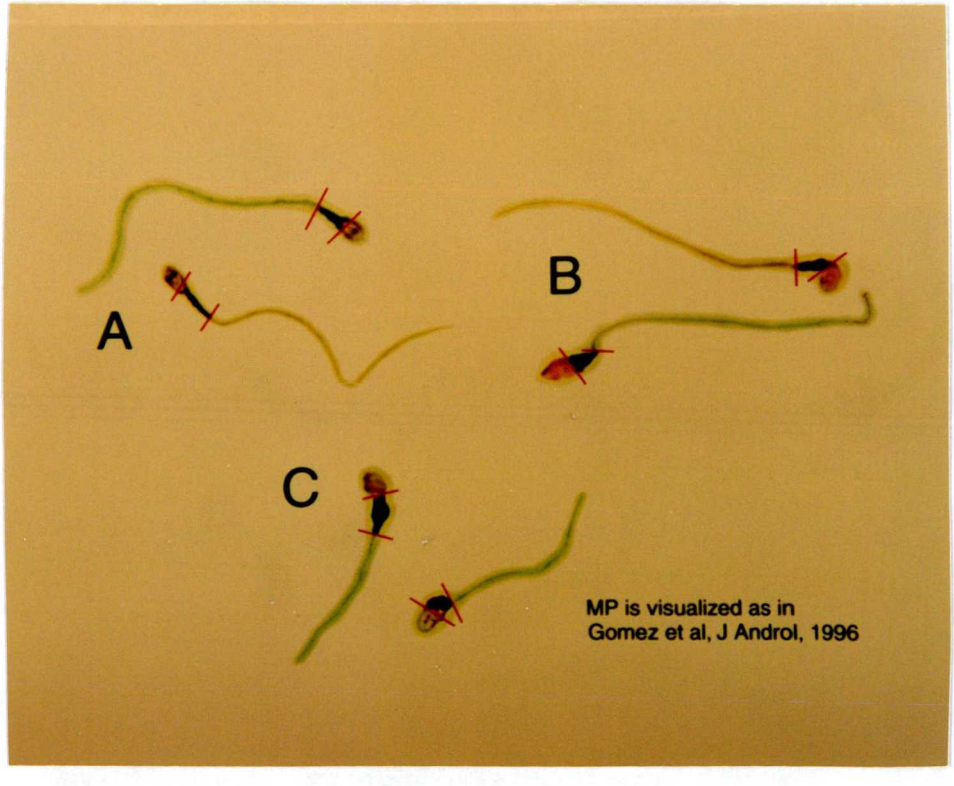

FIGURE 7b.

TABLE I
MORPHOMETRY OF SPERM HEAD, MIDPIECE AND TAIL
(MEAN±SEM)

	LCKM (N=8 men, 600 sperm in A+B+C)						HCKM (N=12 men, 909 sperm in A+B+C)					
	A (N=201)		B (N=211)		C (N=188)	AV	A (N=322)		B (N=320)		C (N=267)	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	*
H 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
H 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
H 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
H 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
H 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
MP 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	*
MP 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
MP 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	*
MP 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	*
MP 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	*

H: head, MP: midpiece, AV: analysis of variance

* $p<0.001$, § $p<0.05$, ab, ac and bc: significant post-hoc comparisons, NS: Not significant

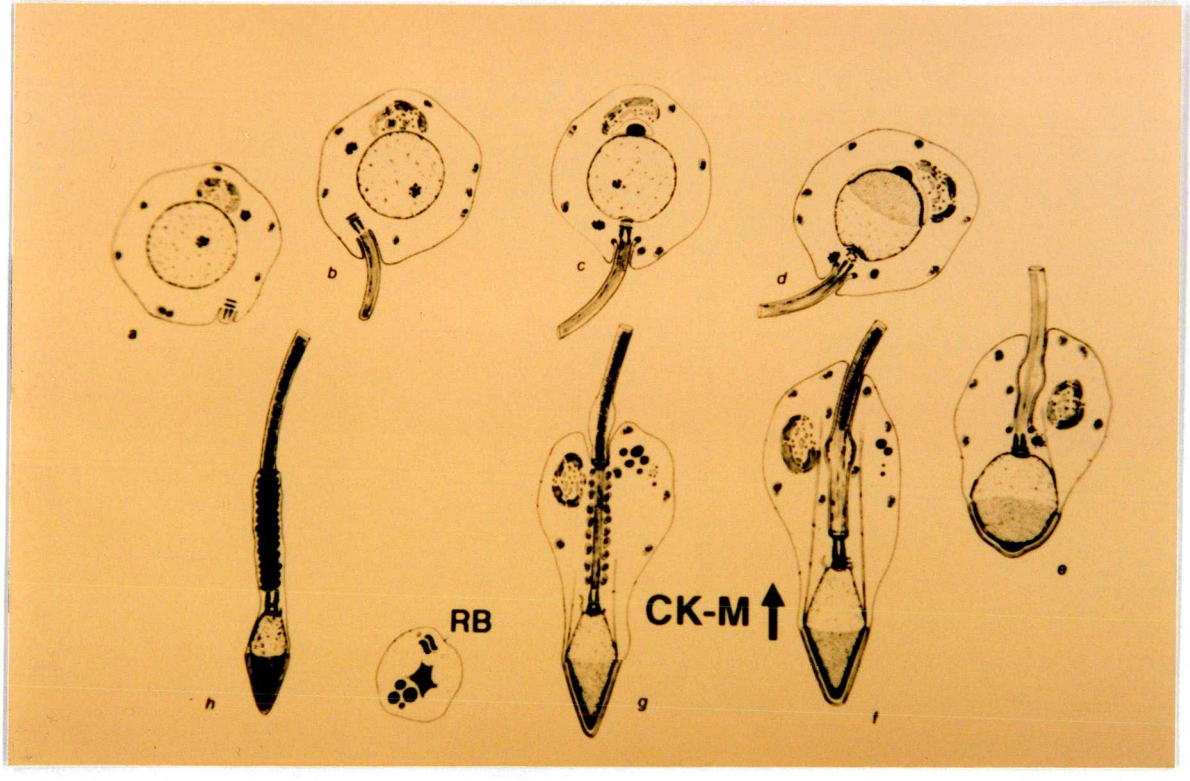

FIGURE 8.