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Article

Biochemical markers of sperm function: male fertility and sperm selection for ICSI

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Abstract

The expression of a 70 kDa chaperone protein, HspA2 (formerly called CK-M), has been identified in mature human spermatozoa. The central role of HspA2 has been established, as the expression level of this protein is related to sperm cellular maturity, DNA integrity, chromatin maturity, chromosomal aneuploidy frequency and sperm function, including fertilizing potential. The spermiogenetic events of cytoplasmic extrusion and remodelling of the plasma membrane, which facilitate the formation of zona pellucida binding site(s) in human spermatozoa, are related. Finally, the presence of the hyaluronic acid (HA) receptor on the plasma membrane of mature sperm coupled with the HA-coated slide sperm-binding assay, facilitates the testing of infertile men and the selection of single mature spermatozoa for ICSI. Because mature spermatozoa have no residual cytoplasm, the HA-bound sperm fraction is also enriched in spermatozoa that are normal by the Kruger strict morphology method.

Keywords: *chromosomal aneuploidies, human sperm function, hyaluronic acid binding, ICSI sperm selection, maturity, strict morphology*

Introduction

Much research is being focused on the objective biochemical markers of sperm maturity and function. In the past 3 years, advances have been made on the clinical application of the hyaluronic acid (HA) binding assay of human spermatozoa, a test of sperm maturity and fertilizing potential, which also reflects DNA integrity and aneuploidy frequency. Sperm–HA binding also facilitates the selection of individual mature spermatozoa for intracytoplasmic sperm injection (ICSI). The following key points will be discussed: (i) cytoplasmic retention as an evidence of sperm immaturity, and the two-wave expression pattern of the testis-specific HspA2 chaperone protein during meiosis and late spermiogenesis; (ii) cellular maturation, plasma membrane remodelling and their contributions to fertilization function of human spermatozoa; (iii) relationship between sperm immaturity and increased frequencies of chromosomal aneuploidy; (iv) relationship between diminished sperm cellular maturity, persistent histones and diminished DNA integrity; (v) semen analysis and assessment of sperm maturity by HA binding in a double chamber device; (vi) relationship between HA-binding ability of spermatozoa and Kruger strict morphology parameters; and (vii) selection of mature individual spermatozoa with low levels of chromosomal aneuploidy and high DNA integrity.

Biochemical markers of sperm cellular maturation

The primary interest of this research has been the development of objective biochemical markers of human sperm maturity and function, which would predict male fertility, independently from the traditional semen criteria of sperm concentration and motility. In measurements of sperm creatine-N-phosphotransferase or creatine kinase (CK), significantly higher sperm CK activities have been found in men

with diminished fertility (Huszar *et al.*, 1988a,b). The research has addressed reasons underlying the sperm CK activity differences by direct visualization of the CK in individual spermatozoa with CK immunocytochemistry (Huszar and Vigue, 1993). The autoradiographic and CK immunostaining patterns indicated that the high sperm CK activity was a direct consequence of increased cytoplasmic protein and CK concentrations in the spermatozoon. The combination of increased CK and protein concentrations, coupled with the diminished fertility, suggested the identification of a sperm developmental defect in the last phase of spermiogenesis when the cytoplasm (unnecessary for the mature spermatozoon) normally is extruded and left in the adluminal area as 'residual bodies' (Clermont, 1963).

Following electrophoretic analysis of human sperm extracts, in addition to the CK-B isoform, another ATP-containing protein was found, which was proportional to the incidence of mature spermatozoa characterized by low CK activity and no cytoplasmic retention in the semen samples (Huszar and Vigue, 1990). This developmentally regulated protein has been identified as the 70 kDa testis-expressed chaperone protein, which in humans is called HspA2 (Huszar *et al.*, 2000). The close inverse correlation between the proportions of spermatozoa with cytoplasmic retention and low expression of HspA2 and those spermatozoa with lack of cytoplasmic retention and increased expression of HspA2 indicated that cytoplasmic extrusion and commencement of the HspA2 synthesis are related, developmentally regulated spermiogenetic events. In three independent studies, the correlation between HspA2 levels and CK activity was $r = -0.69$, -0.71 and -0.76 ($P < 0.001$, $n = 159$, 134, and 119) (Huszar *et al.*, 1990; Lalwani *et al.*, 1996; Ergur *et al.*, 2002). It was established that all sperm maturational events related to the decline of CK activity and increase in HspA2 expression are completed by the time the spermatozoa enter the caput epididymidis (Huszar *et al.*, 1998).

HspA2 which, due to its electrophoretic properties and ATP content, was initially assumed to be an unusual form of sperm specific CK-M isoform (several properties have also indicated that it was not a conventional CK-M; Huszar and Vigue, 1990), proved to be a most useful objective biochemical marker. It has been shown that mature and immature spermatozoa are different with respect to HspA2 ratio, as expressed by the concentrations of sperm CK and HspA2 [%HspA2/(HspA2 + CK-B)], morphological and morphometrical attributes, zona pellucida-binding properties and fertility (Huszar *et al.*, 1992, 1994). Furthermore, it has been established that in spermiogenesis, simultaneously with cytoplasmic extrusion and the commencement of HspA2 synthesis, the sperm plasma membrane also undergoes maturation-related remodelling. This remodelling step facilitates the formation of the sites and receptors for zona binding and for hyaluronic acid binding in mature spermatozoa (Huszar *et al.*, 1997).

Sperm maturity and fertilization function

The predictive value of CK activity, representing cytoplasmic retention, was tested in couples with oligozoospermic husbands treated with intrauterine insemination. In spite of identical sperm concentration and motility parameters in husbands of those couples that have or have not achieved pregnancy, those with pregnancies had four times lower sperm CK activity ($P < 0.001$). In addition, a logistic regression analysis indicated that sperm CK activity, but not sperm concentrations, contributed significantly to the predictive power (Huszar *et al.*, 1990).

The validity of HspA2 ratio in the assessment of male fertility was tested in two blinded studies of couples undergoing IVF. In the first, 84 husbands from two different IVF centres were classified (without any information on their semen parameters or reproductive histories) based only on their sperm HspA2 ratios into 'high likelihood' (>10% HspA2 ratio) and 'low likelihood' (<10% HspA2 ratio) for fertility groups. All pregnancies occurred in the 'high likelihood' group. No pregnancy occurred in the 'low likelihood' group. In the 'high likelihood' group, if at least one oocyte was fertilized, indicating the lack of oocyte defects in the wife, the predictive rate of HspA2 ratio for pregnancy was a very high 30.4% per cycle. An additional important utility of the HspA2 ratio became apparent: nine of the 22 'low likelihood' men were normozoospermic, but had diminished fertility. Thus, the HspA2 ratio provided, for the first time, a diagnostic tool for unexplained male infertility (infertile men with normal semen, Huszar *et al.*, 1992).

Morphometrical differences have also been demonstrated between mature and diminished maturity spermatozoa (Gergely *et al.*, 1999). More recently, the utility of CK-M ratios in predicting IVF failure has been examined in 119 couples treated at Yale. Similar to the 1992 study, none of the 25 men with <10% CK-M ratios was able to father children, whether they had low or high sperm concentrations (Ergur *et al.*, 2002). The value of sperm CK studies has also been confirmed by other laboratories (Gomez *et al.*, 1996; Orlando *et al.*, 1994; Sidhu *et al.*, 1998).

It has also been established that sperm samples with high CK activities and cytoplasmic retention have proportionally higher levels of lipid peroxidation (Aitken *et al.*, 1994; Huszar and Vigue, 1994). Due to the high level of reactive oxygen species, there is increased degradation of DNA, which contributes to the diminished ability of the spermatozoon to provide the paternal contribution to the zygote. The high level of lipid peroxidation has not affected normal spermatozoa without cytoplasmic retention, even if incubated in sperm pellets with high reactive oxygen species producing spermatozoa. For this reason, it has been concluded that the high level of lipid peroxidation in spermatozoa is an 'inborn' error, rather than an 'acquired' property (Huszar and Vigue, 1994). In a recent review, the factors that characterize immature spermatozoa with defective function, such as cytoplasmic retention and consequential abnormal sperm morphology, high level of lipid peroxidation, DNA fragmentation and aneuploidies, were cited as key elements in the aetiology of human infertility and genetic mutations in the offspring (Aitken *et al.*, 2003). It is of interest that the potential relationship between diminished sperm maturity and aspects of male germ cell apoptosis is not yet understood (Henkel *et al.*, 2003; Oehninger *et al.*, 2003).

To identify the steps in the fertilization process at which the low HspA2 immature spermatozoa are deficient, human sperm–oocyte binding was explored. With the study of sperm–hemizona complexes, it was established that only the

clear headed (low CK), mature spermatozoa were able to bind to the zona (Huszar *et al.*, 1994; **Figure 1**). Spermatozoa with retained cytoplasm were deficient in the oocyte binding site. In a further study, it was confirmed that plasma membrane remodelling occurs in human spermatozoa, simultaneously with cytoplasmic extrusion, during spermiogenetic maturation. This was demonstrated by the close correlation (r = 0.8) between CK concentration or the HspA2 ratio and the density of the sperm plasma membrane-specific enzyme $\beta_{1,2}$-galactosyltransferase in sperm fractions of various maturities (Huszar *et al.*, 1997). Such remodelling apparently facilitates the formation of the zona pellucida- and hyaluronic acid-binding sites. This finding explains two major characteristics of spermatozoa with diminished maturity: cytoplasmic retention and deficiency in zona pellucida binding (Huszar and Vigue 1993, 1994; Huszar *et al.* 2000).

In general, chaperone proteins facilitate the assembly and intracellular transport of proteins. Indeed, the expression of HspA2 is simultaneous with major sperm protein movements underlying cytoplasmic extrusion and remodelling of the human sperm plasma membrane. This in turn facilitates the development of zona pellucida-binding site(s). It is thought that retention of the cytoplasm, and the lack of zona-binding sites in immature spermatozoa, are probably related to the diminished expression of HspA2, and also to diminished DNA integrity, as a consequence of the impaired delivery of DNA repair enzymes during and following meiosis. In order to confirm findings regarding the expression of HspA2 during terminal spermiogenesis, the expression pattern of HspA2 in human testicular tissue was also examined (**Figure 2**). Varying low levels of immunostaining were evident in spermatocytes and spermatids, reflecting the presence of HspA2, which was also identified in mouse spermiogenesis in the synaptonemal complexes. However, the staining was particularly striking in the cytoplasm of elongating spermatids and mature spermatozoa about to be released from the adluminal compartment (Huszar and Vigue, 1990; Huszar *et al.*, 2000).

From the perspective of male infertility, it is important that synthesis of the 70 kDa family of testis-specific chaperone proteins is developmentally regulated. In the mouse, the chaperone protein homologous to HspA2 is the Hsp70-2, which arises from a different gene. Another member of this family is hsc70, which in the mouse is expressed in terminal spermiogenesis, and has not yet been identified in human testis (Eddy, 2002). Hsp70-2 in mice appears during meiotic prophase as a component of the synaptonemal complexes. The apparent functions of Hsp70-2 are maintaining the synaptonemal complexes and assisting chromosome crossing over during meiosis and spermatocyte development (Allen *et al.*, 1996). Accordingly, the targeted disruption of the *hsp70-2* gene causes arrested sperm maturation and azoospermia (Dix, 1997). These events could be related to faulty meiotic recombination in spermatocytes, disruption of the meiotic cell cycle regulatory machinery, or perhaps to a more direct disruption of the apoptotic machinery in spermatocytes or even in spermatids or ejaculated immature spermatozoa (Dix, 1997). Regarding human spermatozoa, this was the first demonstration of the expression pattern of the HspA2 protein in human testis and spermatozoa and correlation of the expression level of HspA2 to sperm function (Huszar *et al.*, 2000). Because maturational differences in cytoplasmic content, plasma membrane remodelling, DNA integrity and aneuploidy rates had already been identified, subsequent studies explored whether the plasma membrane structure differences and features specific for mature spermatozoa could facilitate the selection of mature spermatozoa for ICSI.

Diminished sperm maturity: DNA integrity and chromosomal aneuploidies

Because HspA2 is a component of the synaptonemal complex, it was postulated that the frequency of chromosomal aneuploidies will be higher in immature versus mature spermatozoa (Kovanci *et al.*, 2001). This question has been examined in spermatozoa from semen and from 80% Percoll pellets (enhanced in mature spermatozoa) of the same ejaculate in 10 oligozoospermic men. Immature spermatozoa with retained cytoplasm, which signifies spermiogenetic arrest, were identified by immunocytochemistry. Approximately 7000 sperm nuclei were evaluated with fluorescence in-situ hybridization (FISH) in each of the 20 fractions (142,086 spermatozoa in all) using centromeric probes for the X, Y, and 17 chromosomes. The proportions of immature spermatozoa (as detected by cytoplasmic retention) were 45.4 ± 3.4 versus 26.6 ± 2.2% in the two groups (medians: 48.2 versus 25%, $P <$ 0.001, n = 300 spermatozoa evaluated per fraction, 6000 spermatozoa in all). There was also a concomitant decline in total disomy, total diploidy and total aneuploidy frequencies in the 80% Percoll versus semen fractions (0.17 versus 0.54%, 0.14 versus 0.26% and 0.31 versus 0.81%, respectively, $P <$ 0.001 in all comparisons). The mean decline of aneuploidies was 2.7-fold. Regarding the hypothesis that aneuploidies are related to sperm immaturity, there was a close correlation between the incidence of immature spermatozoa and disomies ($r = 0.7$, $P < 0.001$), indicating that disomies originate primarily in immature spermatozoa. Thus, the idea that the common factor underlying sperm immaturity and aneuploidies is the diminished expression of the HspA2 appears to be valid (Huszar *et al.*, 2000; Kovanci *et al.*, 2001).

In another study, it was found that the elimination of disomic spermatozoa with diminished maturity was more effective with gradient centrifugation, whereas spermatozoa with diploidy were more reduced in semen fractions prepared by the swim-up approach (Jakab *et al.*, 2003).

Association between chromosomal aneuploidies and nuclear immaturity

As discussed, during spermiogenesis there are concurrent nuclear and cytoplasmic processes in the developing male germ cell, including histone–protamine replacement, cytoplasmic extrusion, plasma membrane remodelling and formation of the acrosome and tail.

Studies have looked for a relationship between numerical chromosomal aberrations and persistent histones, potentially indicating that errors in the spermatogenetic and spermiogenetic phases simultaneously occur in immature spermatozoa.

In seven moderately oligozoospermic men, an average of 8399 spermatozoa (58,793 spermatozoa in all) were evaluated for aniline blue staining pattern as light (L, most mature spermatozoa), intermediate (IN) and dark (D, immature spermatozoa). The FISH patterns of sperm nuclei were scored by strict criteria. The images of the aniline blue-treated spermatozoa were digitized and saved, along with their field locations on the slides, using the Metamorph program (Universal Imageing Co., PA, USA) for identification following FISH step as spermatozoa having normal, disomic or diploid nuclei. It was found that immature sperm cells with D aniline blue histone staining showed no FISH signals. It is unclear whether this was due to a failure of DNA uncoiling during decondensation, or to a high degree of DNA degradation, which is known to occur in immature spermatozoa. The spermatozoa with L and IN patterns showed normal decondensation and easily evaluable FISH signals. There was an aggregate five-fold increase in the frequency of disomies in the IN versus the mature L pattern spermatozoa. The relationship between disomies and nuclear immaturity was further emphasized by the close correlation between proportions of spermatozoa with the IN pattern and total disomy rates ($r = 0.76$, $P < 0.05$; Óvári *et al.*, 2003).

These results, using double nuclear detection probes of human spermatozoa, indicate that diminished maturity spermatozoa show defects in both the spermatogenetic and spermiogenetic phases of male germ cell development. The close relationship between occurrence of persistent histones and chromosomal disomies further points to the common origin of the defects: low expression levels of the HspA2 chaperone protein.

Sperm–HA-binding assay: a test of sperm maturity and fertility

Current ideas on sperm maturation in men are summarized in **Figure 3**. Looking for the reason underlying for diminished zona binding by immature spermatozoa, it has been established that in spermiogenesis, simultaneously with cytoplasmic extrusion and the commencement of HspA2 synthesis, the sperm plasma membrane also undergoes maturation-related remodelling that promotes the formation of zona-binding and HA-binding sites. Thus, in immature spermatozoa with cytoplasmic retention, there is a low density of zona-binding sites and also of HA receptors (Huszar *et al.*, 1997, 2003).

Based on the above concepts, three questions were examined. Firstly, would spermatozoa bind permanently to solid state hyaluronic acid? Secondly, the diagnostic utility of sperm binding to HA was examined, in a double chamber device in which the A side provided the measures of sperm concentration and motility (thus motile sperm concentration), and the B side was coated with HA in order to test the proportion of mature sperm exhibiting HA binding. Finally, the potential correlation between sperm CK activity or sperm HspA2 expression (proven clinical utility in predicting diminished fertility), increase in the proportion of sperm with strict morphology within the HA-bound sperm fraction, and the rate of sperm binding to HA were studied. The results are described below.

Sperm binds to HA

There were three sperm populations: (i) spermatozoa permanently bound to HA; (ii) spermatozoa exhibiting no binding; (iii) a small proportion of spermatozoa (<5%) that has initially bound to HA, was released shortly, and rebound again. These three patterns were interpreted as mature spermatozoa with high density HA receptor, immature spermatozoa with deficient maturity and plasma membrane remodelling, and spermatozoa of intermediate maturity with a low density of HA receptors (**Figure 4**). The specificity of HA binding has been determined by two methods. First, HA was left out from the slide coating mixture. Spermatozoa no longer bound to the slide. Using various polymers, similar to HA, in the coating mixture did not result in sperm binding. Adding HA to the solution, thus saturating the HA receptors, caused a decline in binding efficiency. Unfortunately, no specific antibodies to the HA receptor are yet available, thus it was impossible to perform definitive binding inhibition experiments. These experiments will be performed as soon as the reagents are available (Huszar *et al.*, 2003).

In the HA-binding experiments, points of further interest have been found. If the spermatozoa were non-viable, they did not bind to HA. Thus, membrane integrity is closely related to binding ability. The potential relationship between HA binding and acrosomal integrity has also been explored, by following the FITC–*Pisum sativum* fluorescence pattern. The data indicate that spermatozoa with intact or slightly reacted acrosomal cap are able to bind to HA. However, spermatozoa with a further advanced activation state are non-binders. Thus it appears, in line with the top of the cap binding pattern, that the HA receptors are localized on the acrosomal membrane.

Correlation of binding with sperm maturity markers

When the relationship between sperm binding and CK activity ($n = 50$ men) was studied, a strong correlation ($r = -0.56$, $P < 0.001$) was found, which is a well-characterized sperm maturity marker (**Figure 5**). The fact that the correlation was closer with CK activity, as compared with the HspA2 ratio (data not shown), is an expected effect because the membrane remodelling and the formation of the HA receptor sites occur simultaneously with cytoplasmic extrusion, whereas HspA2 expression occurs in two waves (in meiotic spermatocytes as a part of the synaptenomal complex and in terminal spermatogenesis when the cytoplasm is extruded); thus it is not directly related to membrane remodelling.

Diagnostic utility of sperm binding to HA

Finally, CK activities, HspA2 ratios and per cent binding of spermatozoa to HA-coated slides were evaluated in 46 men. With respect to binding, sperm populations were classified as follows: >90% ($n = 26$) were excellent, between 60–90% ($n = 12$) were intermediate, and <60% ($n = 8$) were diminished binders. In line with previous findings with respect to CK and HspA2, the sperm binding scores were largely independent from sperm concentration. Among men within the $<20 \times 10^6$ spermatozoa/ml concentration range ($n = 14$ of 56 men), three excellent, five moderate, and six diminished HA binders were identified.

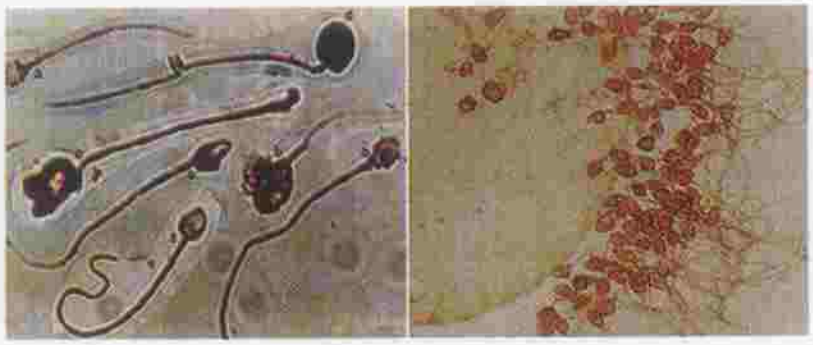

Figure 1. *Left*, panel of immature/mature sperm after CK immunostaining of the retained cytoplasm. *Right*, CK immunostained sperm–hemizona complex. Observe that only the clear headed mature spermatozoa without cytoplasmic retention are able to bind.

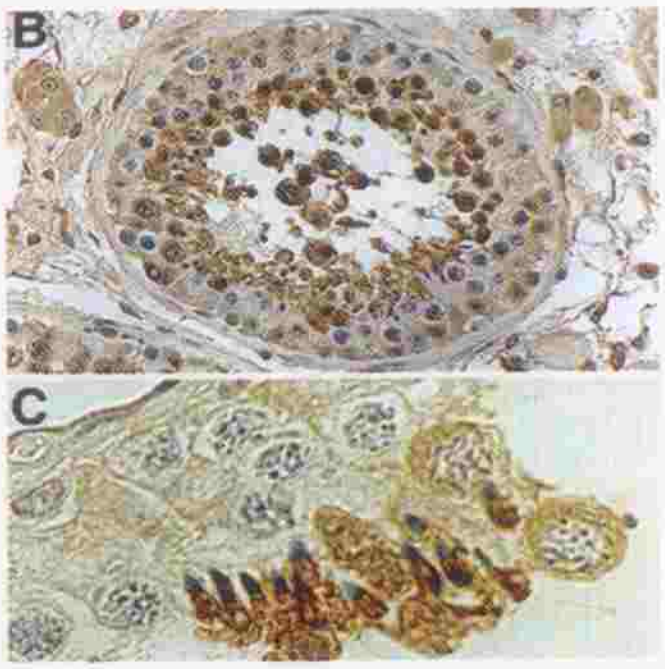

Figure 2. Human testicular biopsy tissues immunostained with human HspA2 antiserum. Sections B and C in the composites represent different magnifications to illustrate the tubular structure, and the staining pattern of the adluminal area. HspA2 expression begins in meiotic spermatocytes, but is predominant during terminal spermiogenesis in the elongated spermatids and spermatozoa.

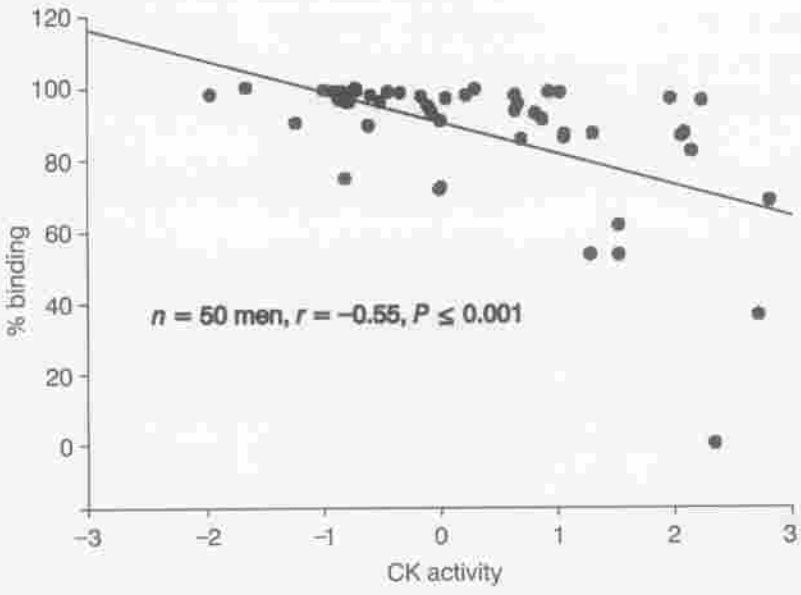

Figure 5. Correlation between HA binding and CK activity.

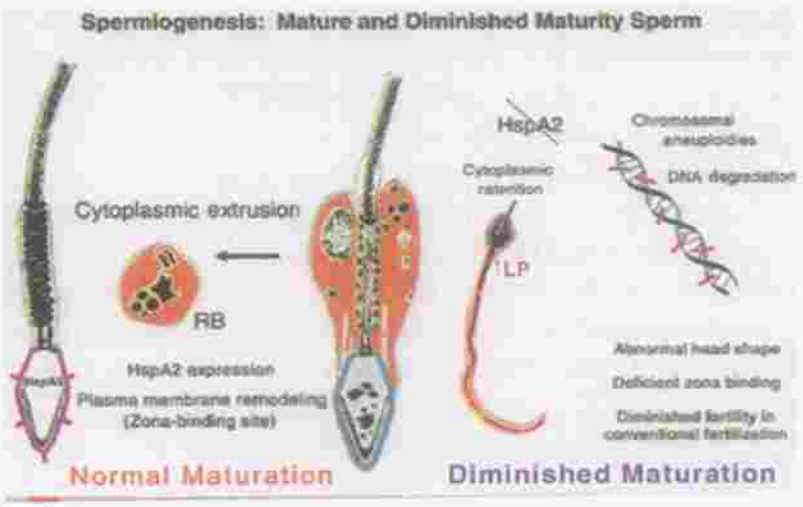

Figure 3. A model of normal and diminished maturation of human spermatozoa. In normal sperm maturation, HspA2 is expressed in the synaptonemal complex of spermatocytes, supporting meiosis. HspA2 is probably also involved in the processes of late spermiogenesis, such as cytoplasmic extrusion (represented by the loss of residual body, RB), plasma membrane remodelling, and the formation of the zona pellucida-binding site (change from the blue to the red membrane and the stubs). Diminished maturity spermatozoa lack HspA2 expression, which causes meiotic defects and a higher rate of retention of CK and other cytoplasmic enzymes, increased levels of lipid peroxidation (LP) and consequent DNA fragmentation, abnormal sperm morphology and deficiency in zona-binding and HA-binding sites.

Figure 4. Sperm movement patterns in the double chamber device. A side: uncoated glass chamber with patterns of motile sperm. B side: mature spermatozoa are bound, and diminished motility spermatozoa remain motile in the HA-coated chamber. Spermatozoa are stained with Cyber green DNA stain (Molecular Probes, Eugene, Oregon) that permeates viable spermatozoa.

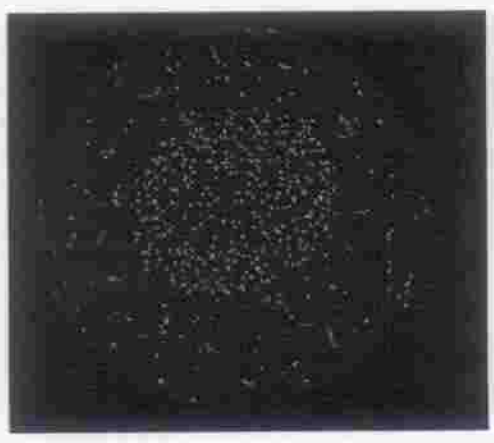

Figure 6. Spermatozoa approach from the periphery, and then bind to the HA spot on a Petri dish.

Sperm–HA binding and strict morphology

Based on current understanding of sperm cellular maturity, it was postulated that HA-bound spermatozoa will show an improved strict morphology score. Twenty-four men were studied (concentration 57.2 ± 10.4 × 10^6 spermatozoa/ml; motility: 55.3 ± 2.8%, all data mean ± SEM). For the HA-binding assay, CellVu double chamber slides were used. On the A side, motile sperm concentration was assessed. HA binding was measured on the HA-coated B side, by calculating the percentage of spermatozoa that remained unbound after 15 min incubation. Semen was added to the A and B sides of another slide, and after 15 min the non-bound spermatozoa on the B side were gently washed off with human tubal fluid (HTF). Spermatozoa on both sides were fixed and stained with DiffQuik. Kruger strict morphology was performed on spermatozoa from all 24 men by two investigators who evaluated 200 spermatozoa on both the A and the B sides (Zavaczki *et al.*, 2003).

The proportion of normal spermatozoa was higher in the HA-bound population on the B side as compared with semen on the A side (18.1 ± 1.3% versus 7.8 ± 0.8% $P < 0.001$, $n = 400$ spermatozoa evaluated in each man). The mean strict morphology score improvement on the B versus A sides was 2.6 ± 1.0-fold (range: 1.6–6.3). The proportion of spermatozoa that did not bind to HA was related to the improvement in morphology on the B versus the A side ($r = 0.48$, $P < 0.02$).

The HA-binding ability of spermatozoa is related to sperm maturity and Kruger strict morphology. There was a significant improvement in the proportion of normal spermatozoa in the HA-selected population. HA binding was also related to the objective morphometry attributes that have been shown to reflect sperm maturity, as measured by the biochemical markers of CK activity and HspA2 ratio (Gergely *et al.*, 1999). The relationship with strict morphology further indicates that the sperm–HA-binding test is a quick and effective assessment that may be used in the offices of physicians treating male infertility.

Selection of spermatozoa with low aneuploidy frequencies for ICSI

Previously, it was found that mature, but not immature, spermatozoa in response to HA showed increased velocity and retention of long-term motility (Sbracia *et al.*, 1997). It was suggested that this effect was receptor mediated. Based on the association between sperm maturation and plasma membrane remodelling, it has been suggested that the presence of the HA receptor in mature, but not in immature spermatozoa, and a respective device with HA-coated surface, will facilitate the selection of single mature spermatozoa with high DNA integrity and low frequency of chromosomal aneuploidies for ICSI (Huszar *et al.*, 2003).

As discussed, there is a relationship between the proportion of immature spermatozoa with cytoplasmic retention and frequency of chromosomal aneuploidies in human spermatozoa (Kovanci *et al.*, 2001). This relationship is based on the dual role of the HspA2 chaperone, which supports meiosis as a component of the synaptonemal complex, and facilitates plasma membrane remodelling as well as the formation of the zona pellucida and HA-binding sites during spermiogenesis (**Figure 3**). The increased rate of chromosomal aberrations and other potential consequences of using immature spermatozoa for ICSI are of major concern. Data have been presented to show that HA-selected mature spermatozoa show a low frequency of chromosomal aberrations comparable to that of sperm selected by the zona pellucida in conventional fertilization. HA is a normally occurring component of the female reproductive tract; thus there should not be any ethical concerns (**Figure 6**).

In ongoing studies, the efficiency of sperm selection with respect to elimination of spermatozoa with chromosomal aneuploidies and diploidies has been tested. Washed spermatozoa of eight moderately oligozoospermic men (OS, sperm concentration ± SEM: 20.6 ± 1.7 × 10^6/ml, motility: 54.1 ± 2.5%) and 80% isolate gradient sperm pellets from seven normozoospermic IVF patients (ISL80, sperm concentration 118 ± 21.4 × 10^6/ml, motility: 59.1 ± 4.9%) were studied. Spermatozoa suspended in HTF were placed over HA spots bonded to Petri dishes (Biocoat Co., PA, USA). After incubation for 15 min, the HA-attached spermatozoa were collected using an ICSI micropipette. Aliquots of the sperm suspension and HA-bound spermatozoa were examined after FISH, using centromeric probes for the X, Y and 17 chromosomes. Data were analysed by chi-squared analysis.

In each man, the initial sperm suspension (mean: 3000 spermatozoa, 45,000 spermatozoa in the 15 men) was evaluated, and all HA-bound spermatozoa collected in the eight OS men (mean: 753, range: 224–1142) and seven ISL80 men (mean: 644, range 224–1128). In the OS group, the proportion of disomies showed a mean 6.9-fold reduction, and the sex chromosome disomies alone a 3.6-fold reduction. Diploidies in the HA-selected samples were 6.4-fold lower compared with the initial semen sample. With respect to the ISL80 group, the disomy rates declined in the HA-bound fractions (**Table 1**). The decrease for sex chromosomes was approximately four-fold, even though that the ISL80 samples were 80% isolate pellets of normozoospermic men (thus an 'ideal sperm' fraction). The incidence of diploid sperm decreased six-fold in both groups ($P < 0.001$).

It can be concluded that HA selection eliminated spermatozoa with disomy and diploidy. The four-fold decline of sex chromosome disomies is consistent with the increase of

Table 1. Reduction of sperm nuclei with disomy and diploidy in the HA-bound fractions.

	Group OS		
	Disomy		*Diploidy*
	Sex	*17*	
Initial (%)	0.35	0.23	0.81
HA-bound (%)	0.09	0.04	0.13
Reduction	4.0×	5.3×	6.1×
P (chi-squared)	<0.001	<0.001	<0.001

OS = oligozoospermic men.

chromosomal aberrations in ICSI children. In spite of the sample differences, the aneuploidy and diploidy rates in the HA-bound fraction declined to a narrow and low 0.04–0.13% range, which is comparable to normal fertile men. Thus, HA sperm selection provides a new, safe and efficient method for selection of mature spermatozoa for ICSI.

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