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Review

Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects

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Abstract

The testis-expressed chaperone protein, HspA2 (previously creatine kinase M isoform) was established as a measure of human sperm cellular maturity, function and fertility. The presence of HspA2 in the synaptonemal complex is likely to link low HspA2 expression and increased frequency of chromosomal aneuploidies in arrested-maturity spermatozoa. A relationship also exists between HspA2 expression in elongating spermatids and the associated spermatogenetic events, including plasma membrane remodelling and the formation of zona pellucida and hyaluronic acid (HA) binding sites. The HA receptor of mature spermatozoa, when coupled with HA-coated slides and/or Petri dishes, allows visual observation of sperm–HA binding, providing a basis for sperm maturity testing, a major improvement in semen evaluation, and selection of mature spermatozoa for intracytoplasmic sperm injection (ICSI). Thus, in HA-selected spermatozoa the frequency of chromosomal disomy and diploidy is reduced 4- to 6-fold compared with semen sperm fractions. This reduction is similar to the increase in numerical chromosomal aberrations in ICSI children. Combined studies of sperm shape and chromosome probes demonstrated that sperm morphology does not aid selection of haploid spermatozoa. The HA-mediated sperm selection is a novel and efficient technique that may alleviate potential problems related to ICSI fertilization with visually selected spermatozoa.

Keywords: *sperm maturity, biochemical markers, chromosomal aneuploidies, DNA fragmentation, hyaluronic acid binding, ICSI*

Introduction

In the past two decades, major advances have been made regarding the biochemical markers of human sperm maturity and function (Cayli *et al*., 2003a; Huszar *et al*., 2006a,b). In this article, a comprehensive overview of the field is presented, including clinical application of the markers, cell biology of sperm maturity and genetic integrity, relationship between sperm shape, chromosomal aneuploidies and sperm hyaluronic acid (HA) binding, and selection of mature spermatozoa for intracytoplasmic sperm injection (ICSI).

Initially, the search was for an objective biochemical parameter that might predict sperm fertility, independently from the classical semen parameters. In assessments of B-type creatine kinase (CK), significantly higher sperm CK content was found in subfertile men (Huszar *et al*., 1988a,b; Cayli *et al*., 2003a). The CK-immunostaining patterns indicated that the high sperm CK activity was a direct consequence of increased cytoplasmic protein and CK concentrations (Huszar and Vigue, 1993). This suggested a sperm developmental defect in terminal spermiogenesis when

the surplus cytoplasm is normally extruded from elongating spermatid as 'residual bodies' (Clermont, 1963).

Another sperm protein with ATP content has also been found in mature spermatozoa (Huszar and Vigue, 1990). This protein was later characterized as the 70 kDa testis-expressed heat shock protein HspA2 chaperone (Huszar *et al.*, 2000). Thus, the proportional concentrations of CK and HspA2 (or HspA2 ratio, formerly CK-M ratio) reflect the proportion of mature and immature spermatozoa in semen samples (Huszar and Vigue, 1990). In three studies (n = approximately 500 men), there were similarly tight correlations between sperm CK activities and HspA2 ratios (r = –0.69, –0.71 and –0.76, $P < 0.001$, n = 159, 134, and 194; Huszar and Vigue, 1993; Huszar *et al.*, 1992; Lalwani *et al.*, 1996). The proportion of mature and immature spermatozoa, whether the men were normozoospermic or oligozoospermic, showed a day-to-day and man-to-man variation in semen samples (Huszar *et al.*, 1988b). Indeed, in a study of couples treated with intrauterine insemination, a logistic regression analysis indicated that sperm CK activity did, but sperm densities did not, provide predictive values for pregnancies in oligozoospermic men (Huszar *et al.*, 1990a; Huszar *et al.*, 1988b, Huszar and Vigue, 1993).

In further studies of sperm–hemizona (halved unfertilized human oocytes) complexes, all zona pellucida-bound spermatozoa were clear-headed without cytoplasmic retention. Thus, immature spermatozoa with retained cytoplasm, and low expression of HspA2, were deficient in the zona-binding site (Huszar *et al.*, 1994). The hypothesis was examined that during spermiogenesis, a plasma membrane remodelling step also occurs. In a blinded study, focusing upon β1,2-galactosyltransferase (present exclusively in the sperm plasma membrane), the cytoplasmic content of CK or HspA2 concentrations and the membrane density of β1,2-galactosyltransferase were closely related (both $r > –0.8$ or $r > 0.8$ respectively, both $P < 0.001$; Huszar *et al.*, 1997). Thus, during normal spermiogenesis and plasma membrane remodelling, along with formation of the zona pellucida binding site, the formation of the hyaluronic acid (HA) binding site also occurs, which is the basis for fertility testing and ICSI sperm selection in men.

Spermatogenetic maturation and sperm fertilizing function

In addition to the clinical utility of sperm CK activity (Huszar *et al.*, 1990a), sperm HspA2 concentrations were also tested in blinded studies of IVF couples. In one study, 84 male partners from the Jones Institute and Yale, USA with known sperm HspA2 concentrations; other semen parameters or reproductive histories were unknown) were assigned into 'high likelihood' (>10% HspA2) and 'low likelihood' (<10% HspA2) for fertility subgroups (Huszar *et al.*, 1992). All pregnancies were found in the 'high likelihood' group. An additional benefit of the HspA2 concentrations has also become apparent: nine of the 22 'low likelihood' men were normozoospermic but had arrested sperm maturity and <10% HspA2. Thus, the HspA2 provides a diagnostic utility for unexplained male infertility (infertile men with normal semen; Huszar *et al.*, 1992). Recently, HspA2 was re-examined in 194 couples treated with IVF at Yale. The receiver operating characteristic (ROC) analysis showed a 100% predictive value for failure to achieve pregnancy in the range of <10.4% HspA2 threshold. As in the 1992 study, nine of the 15 men with <10% HspA2 ratio and pregnancy failure were normozoospermic (Ergur *et al.*, 2002).

From the perspective of male gamete maturation, it is important that synthesis of the HSP70 family of proteins (HspA2 in men) is regulated developmentally, and that they contribute to the maintenance of the synaptonemal complex and to the meiotic function during spermatocyte development (Allen *et al.*, 1996; Eddy, 1999; Son *et al.*, 1999; Tsunekawa *et al.*, 1999). In line with these functions, the targeted disruption of the *hsp70–2* gene in mice caused arrested spermatogenesis and azoospermia (Dix *et al.*, 1996). With respect to human spermatozoa, the authors' laboratory was the first to demonstrate the two-wave expression of HspA2 (**Figure 1**), first in spermatocytes as a meiotic component within the synaptonemal complex, second in elongating spermatids during terminal spermiogenesis (Huszar *et al.*, 2000). In further human and stallion studies, it was found that all CK and HspA2-related maturational events are completed in sperm cells that descended to the caput epididymidis (Huszar *et al.*, 1998b).

In summary, the expression levels of HspA2 are closely related to sperm cellular maturity, and fertilizing potential. Immature spermatozoa with cytoplasmic retention also exhibit increased rates of lipid peroxidation and DNA fragmentation (Aitken *et al.*, 1994; Huszar and Vigue, 1994; Aitken *et al.*, 2003).

Sperm maturation: nuclear and cytoplasmic biochemical markers

The recognition that HspA2 reflects the meiotic process, as well as the cytoplasmic and nuclear down-stream events of cellular maturation in spermatozoa was a key advance (Allen *et al.*, 1996; Eddy, 1999; Tsunekawa *et al.*, 1999; Huszar *et al.*, 2000; Cayli *et al.*, 2003a). The spermatid phase is a dividing point between normally developing and diminished maturity spermatozoa. In elongating spermatids with a substantial up-regulation of HspA2, normal sperm maturation (**Figure 2**, left direction) occurs with the orderly extrusion of cytoplasm in the form of residual bodies, and simultaneous plasma membrane remodelling, yielding a spermatozoon with normal head morphology and fully formed binding sites for the zona pellucida and hyaluronic acid.

In contrast, in spermatids with low HspA2 expression (**Figure 2**, right direction, diminished/arrested maturation), show increased frequencies of chromosomal aneuploidies due to synaptonemal complex defects, as well as DNA chain breaks as a result of inadequate delivery of DNA repair enzymes possibly due to decreased HspA2 chaperone activity (Allen *et al.*, 1996; Eddy, 1999). These cells are characterized by cytoplasmic retention, abnormal head morphology, increased concentrations of reactive oxygen species and consequential further DNA fragmentation. Further, due to the incomplete spermatogenetic process, the plasma membrane remodelling and the formation of the zona pellucida and hyaluronic acid binding sites fail to occur. Thus, arrested maturity spermatozoa are unable to bind to the zona pellucida or fertilize via natural or IVF conception, only by ICSI. The probable reason why such spermatozoa do not die prior to ejaculation is the presence of antiapoptotic protein Bclx2 in the surviving germ cells (Cayli *et al.*, 2004).

Relationship between diminished/arrested sperm maturity and chromosomal aneuploidies

Earlier studies have suggested a relationship between oligozoospermia, male infertility and increased frequencies of chromosomal aneuploidies, (Bernardini *et al.*, 1998; Colombero *et al.*, 1999; Veggeti *et al.*, 2000; Calogero *et al.*, 2001; Templado *et al.*, 2002; Aran *et al.*, 2003; Pang *et al.*, 2005). However, as a novel idea, it is suggested that, because HspA2 is part of the synaptonemal complex and immature spermatozoa are deficient in HspA2, synaptic anomalies may occur in association with arrested maturity (Kovanci *et al.*, 2001). This question has been examined in spermatozoa arising from semen and from 80% Percoll pellets (enhanced in mature spermatozoa) of the same ejaculate. In both sperm fractions of 10 men, approximately 7000 sperm nuclei have been studied with fluorescence in-situ hybridization (FISH; 142,086 spermatozoa in the 10 men), utilizing centromeric probes for the X, Y sex, and 17 chromosomes. The proportions of immature spermatozoa exhibiting cytoplasmic retention were 45.4 ± 3.4 versus 26.6 ± 2.2% in the semen versus Percoll groups (medians: 48.2 versus 25%, $P < 0.001$, $n = 300$ spermatozoa evaluated per fraction, 6000 spermatozoa in all).

There was a close correlation between the incidences of immature spermatozoa with cytoplasmic retention and with spermatozoa of disomic nuclei ($r = 0.7$ with all three chromosomes tested, and $r = 0.76$ with Y disomy, $P < 0.001$ in both), indicating that disomies primarily originate in immature sperm (Kovanci *et al.*, 2001). There was no relationship with diploidies ($r < 0.1$); thus, chromosomal disomy and diploidy are arising from different cellular mechanisms (Egozcue *et al.*, 2002). It was also shown that early and late sperm maturation arrests are related (Salehi-Ashtiani and Goldberg, 1993; Lalwani *et al.*, 1996).

Sperm head shape and sperm maturity: shape or dimensional properties do not facilitate the ICSI selection of haploid spermatozoa

The potential relationship between abnormal sperm morphology and chromosomal aberrations has been of long-term interest (Lee *et al.*, 1996; Yakin and Kahraman, 2001). Earlier data supporting this association were based on the frequencies of abnormal or aneuploid spermatozoa in semen samples. However, examination of the same individual spermatozoa for both attributes finally became possible when it was established that spermatozoa preserve their shape after undergoing the decondensation and denaturation steps that are a prerequisite of FISH analysis (**Figure 3**; Celik-Ozenci *et al.*, 2003).

Based on this finding, the potential role of shape attributes in ICSI sperm selection (Celik-Ozenci *et al.*, 2004) was studied. In these experiments, it was initially established whether spermatozoa were haploid, disomic or diploid with FISH, and the shape and dimensions of the same spermatozoa were subsequently evaluated by their corresponding phase-contrast microscopy images.

First, using objective shape measurements, 1286 individual spermatozoa from 15 men were evaluated: 900 haploid cells, utilizing three-colour FISH for chromosomes 17, X, and Y, and two-colour FISH for the 10 and 11 chromosomes, and all the disomic and diploid sperm images that could be found (368 cells). Second, the 900 non-aneuploid spermatozoa were sorted and classified into three groups as 'small head', 'intermediate head', and 'large head.' Third, the 256 aneuploid and 130 diploid spermatozoa were sorted according to the head size parameter ranges established in the non-aneuploid sperm group, and the frequencies of disomies and diploidies within the 'small', 'intermediate' and 'large' groups were determined.

Aneuploidies and diploidies were present within all three categories. The proportions of the 256 disomic spermatozoa in the small, intermediate, and large sperm head category groups were 66 (27 ± 2%), 56 (23 ± 1%), and 133 (50 ± 2%) respectively. Similarly, the mean number of diploidies in the three sperm head categories were; 3 ± 1, 8 ± 1 and 89 ± 2% respectively. Interestingly, approximately 27% of spermatozoa with disomy and 3% with diploidy of the 386 non-haploid spermatozoa were among the 300 spermatozoa within the small, normal dimensions of the haploid group.

In another analysis of the 1286 images, sperm shape was examined according to their characteristics as 'symmetrical' ($n = 367$), 'asymmetrical' ($n = 368$), 'irregular' ($n = 504$), and 'amorphous' ($n = 47$). Disomic and diploid spermatozoa were present in all four groups with an increasing frequency of 18, 18, 41 and 98% respectively (Celik-Ozenci *et al.*, 2004). Finally, according to the Kruger strict morphology method as normal and abnormal, the proportion of spermatozoa with normal morphology in the symmetrical, asymmetrical, irregular, and amorphous groups were 26, 3, 1 and 0% respectively. There were aneuploid spermatozoa within the Kruger normal group.

Thus, it is evident that visual shape assessment, i.e. choosing the 'best looking' spermatozoon, is an unreliable method for ICSI selection of mature haploid spermatozoa (Celik-Ozenci *et al.*, 2004; Zavacki *et al.*, 2006). This inconsistent relationship between sperm chromosomal aberrations and shape seem to be in contrast with the ICSI sperm selection approach based on enhanced microscopic imaging. However, there are no studies as yet on sperm chromosomal aneuploidies, persistent histones or DNA integrity in spermatozoa selected by the imaging approach (Bartoov *et al.*, 2002: Berkovitz *et al.*, 2006).

Testing of sperm maturity by hyaluronic acid binding in the andrology and IVF laboratories

Concurrently with the studies on sperm maturation, the effects of hyaluronic acid (HA) or hyaluronan on human sperm function were investigated. HA containing medium increased sperm velocity, retention of long-term motility and viability of freshly ejaculated, as well as of cryopreserved/thawed human spermatozoa (Huszar *et al.*, 1990b; Sbracia *et al.*, 1997). These improvements occurred as a response to HA, as indicated by two observations: (i) there was an immediate increase in sperm tail cross-beat frequency and sperm velocity upon HA exposure; (ii) when the HA-exposed spermatozoa, after density gradient centrifugation, were re-suspended in media lacking HA, the

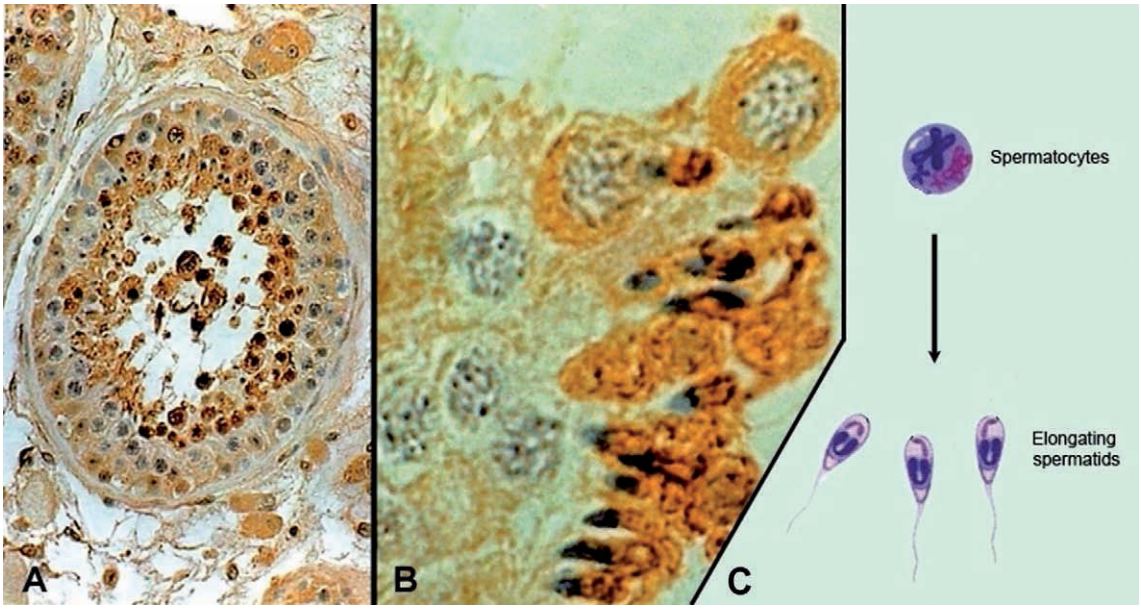

Figure 1. Human testicular biopsy tissue immunostained with heat shock protein HspA2 antiserum. (**A**) illustrates the tubular structure, and the staining pattern of the adluminal area. HspA2 expression begins in meiotic spermatocytes, and there is a second wave of expression in elongating spermatids (**B** and **C**). Modified from Huszar *et al*. (2000).

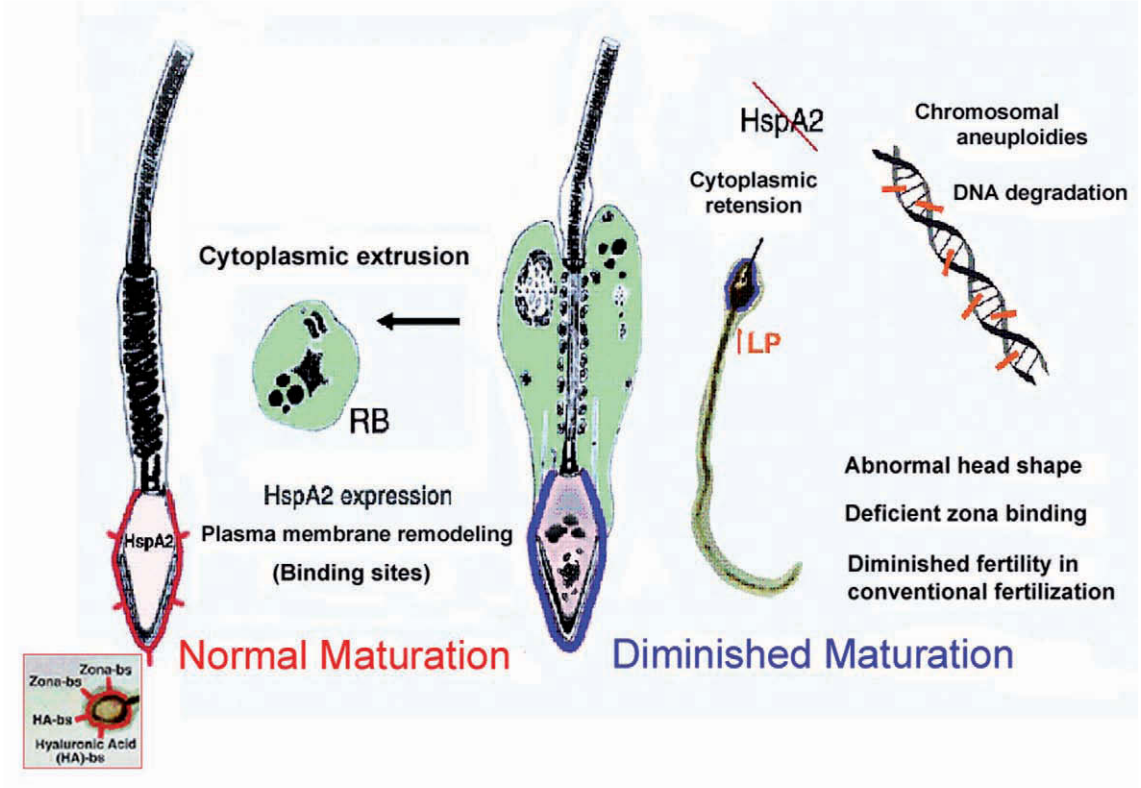

Figure 2. A model of normal and diminished/arrested maturation of human spermatozoa. In normal sperm maturation (left), elongating spermatids undergo cytoplasmic extrusion (represented by the loss of the residual body, RB) and plasma membrane remodelling leading to the formation of the zona pellucida and hyaluronic acid binding sites (bs) (change from blue membrane to red membrane with the stubs). Spermatozoa of arrested maturity have low heat shock protein HspA2 expression, which causes meiotic defects and probably chromosomal aneuploidies. Also, in arrested/diminished maturity sperm there is a higher retention of CK and other cytoplasmic enzymes, increased levels of lipid peroxidation (LP) and consequent DNA fragmentation, abnormal sperm morphology and deficiency in the zona and hyaluronic acid binding sites. Updated from Cayli *et al*. (2003a).

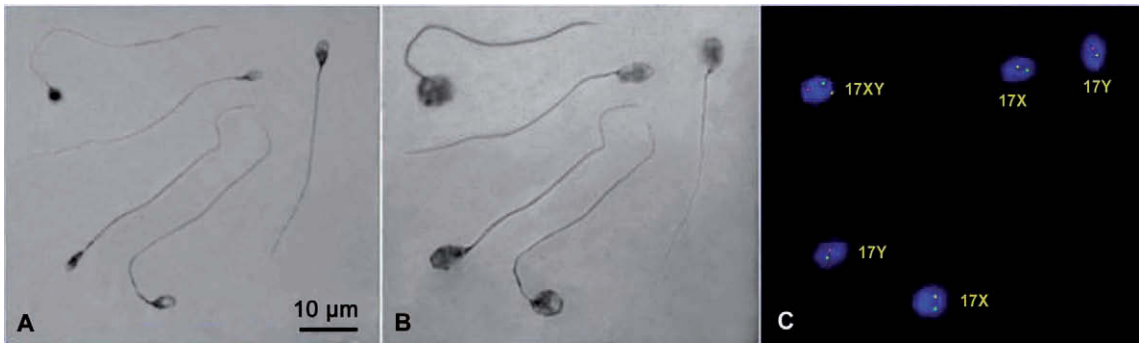

Figure 3. Sperm maintain their shape during the decondensation and denaturation steps necessary for fluorescence in-situ hybridization (FISH). (**A**) Native sperm; (**B**) decondensed sperm. The shape characteristics of sperm in the native and decondensed state are unchanged. (These images are from phase contrast microscopy.) (**C**) fluorescence microscopy of sperm treated with FISH.

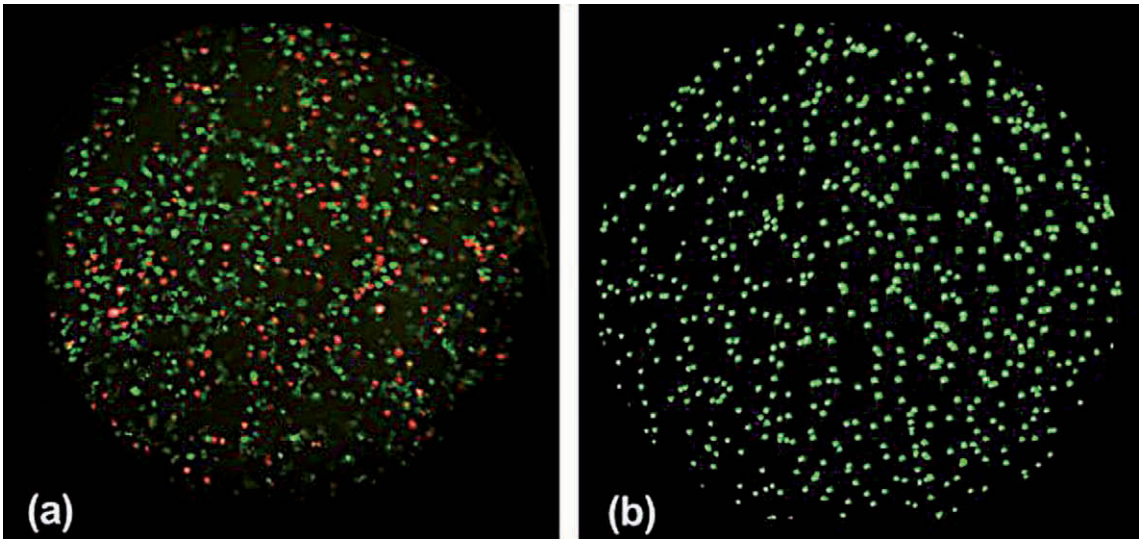

Figure 4. Sperm viability and hyaluronic acid (HA) binding. (**a**) A drop of semen treated with a combination of Cyber Green and propidium iodide, respectively, in order to highlight viable (green) and non-viable (red) spermatozoa. (**b**) HA-bound spermatozoa are gently rinsed, and stained with the same combination of dyes. Only the viable green spermatozoa remain bound to the HA slide. Reprinted from Huszar *et al.* (2003), with permission from the American Society for Reproductive Medicine.

motility and velocity properties returned to those of the control spermatozoa. Thus, the HA effects appear to be receptor mediated (Kornovski *et al.*, 1994; Ranganathan *et al.*, 1994; Cherr *et al.*, 1999). In line with this idea, arrested maturity spermatozoa with retarded plasma remodelling were not stimulated by HA (Huszar *et al.*, 1990b; Sbracia *et al.*, 1997).

It was postulated that: (i) the formation of the HA receptor, similar to the sperm zona-binding site(s), is regulated by the remodelling of the plasma membrane during spermiogenesis; (ii) mature spermatozoa may selectively bind to solid state HA. Indeed, spermatozoa bound to HA were oriented head first, as with sperm zona pellucida binding. This finding is in agreement with the HA binding pattern in monkey spermatozoa (Cherr *et al.*, 1999; Vines *et al.*, 2001). Also, in line with previous hemizona binding experiments, spermatozoa bound to HA exhibited a uniform shape conforming with normal cells of the Kruger classification, which is based on the zona pellucida bound spermatozoa (Kruger *et al.*, 1986; Huszar *et al.*, 1994; Gergely *et al.*, 1999; Celik-Ozenci *et al.*, 2004). Similar data in zona pellucida-bound spermatozoa were reported by others (Liu and Baker, 1992; Liu *et al.*, 2003). In line with the fact that spermatozoa bind head-first to HA with the acrosomal region, spermatozoa with advanced levels of capacitation or acrosome reaction (when the integrity of the acrosomal membrane is disrupted) do not exhibit HA-binding (Huszar *et al.*, 2003).

Further attributes of HA-bound mature spermatozoa indicated that HA-selected spermatozoa are viable (non-viable spermatozoa do not bind, **Figure 4**). HA-bound spermatozoa are devoid of cytoplasmic retention, persistent histones (and probably the associated protamine 1/protamine 2 ratio disorder), DNA fragmentation, and the apoptotic marker caspase 3 (**Figure 5**). These properties are very important because nuclear and cytoplasmic immaturity, particularly the presence of DNA fragmentation, are known to adversely affect the paternal contribution of sperm to the zygote (Huszar *et al.*, 1998a; Aitken *et al.*, 2003; Cayli *et al.*, 2003a, 2004; Celik-Ozenci *et al.*, 2003; Huszar *et al.*, 2003; Aitken 2004; Sati *et al.*, 2004; Seli and Sakkas, 2005; Aioki *et al.*, 2006; Borini *et al.*, 2006).

Relationship between sperm binding to hemizonae and hyaluronic acid

The idea that the HA binding and zona binding sites in spermatozoa are commonly regulated via plasma membrane remodelling was assessed (Huszar *et al.*, 1994, 1997, 2003; Jakab *et al.*, 2005).

The validity of this idea was assessed in a comparative study of sperm–hemizona binding and sperm HA binding scores in aliquots of the same sperm sample in 54 men. By using bisected unfertilized human oocytes and the standard HA-binding slides, there was a close correlation between the binding scores to either HA or hemizona ($r = 0.73$, $P < 0.001$, $n = 54$; Cayli *et al.*, 2003b). Indeed, it is suggested that the correlation is not closer because of the undefined sperm binding ability of unfertilized oocytes, a factor which is more variable than the properties of the uniformly manufactured HA-coated binding slide.

The comparable sperm selection properties of zona pellucida and HA were further confirmed in a collaborative study between the Huszar and Kruger laboratories. In 37 of the 63 samples with <14% Kruger normal teratozoospermic score, the HA-bound spermatozoa versus semen sperm fractions showed an approximately 4-fold enrichment in Kruger normal spermatozoa. The likelihood ratio was 3.04× (95% confidence limits: 1.9×–4.7× considering the three blinded investigators). This enrichment is comparable to the 3.9× improvement reported by Kruger in the zona pellucida bound versus semen spermatozoa (Menkveld *et al.*, 1991; Huszar *et al.*, 2006c). Since sperm–zona pellucida binding is the penultimate fertilization step, sperm HA binding is a very important test. In addition, the HA-coated slide may substitute for the hemizona assay, which is used in only a few laboratories around the world.

A chamber device for the sperm HA binding assay

Based on the fact that mature spermatozoa selectively bind to solid state HA, the sperm HA binding assay has been developed, both as a clinical andrology test, and as a method for selection of mature spermatozoa for ICSI. Sperm binding occurs within 8–10 min. The time-lapse photography highlights the pattern of motile sperm, and arrested motile spermatozoa (see examples in **Figure 6**). The assessment of HA binding is based on the proportions of bound spermatozoa with increased tail cross-beat frequency versus the unbound swimming spermatozoa that do not 'perceive' the HA. Non-motile spermatozoa without tail movement are not considered (Huszar *et al.*, 2003). In line with the inverse relationship between the spermatogenetic events of arrested cytoplasmic extrusion (increased CK retention), and sperm plasma membrane remodelling (formation of the HA binding sites), there was a close correlation between sperm CK activity and HA binding ($r = –0.78$, $P < 0.001$, $n = 56$).

Because the clinical utility of sperm CK activity and HspA2 concentrations were already established in intrauterine insemination (IUI) and IVF studies (Huszar *et al.*, 1990a, 1992; Ergur *et al.*, 2002), a general idea was formulated regarding the expected clinical application of the sperm HA binding score. Three binding zones were considered in 56 men: (i) ≥80% binding (excellent binding, $n = 32$). In these men, the sperm CK activity was 0.18 ± 0.02 CK IU/10^8 spermatozoa (normal range <0.25); thus, there was no male factor infertility or need for intervention; (ii) binding between 60 and 80% (intermediate binding, $n = 14$) (in this group, the sperm CK activity was elevated to 0.50 ± 0.06 CK IU/10^8 spermatozoa). These couples may benefit from IUI. Binding ≤60% (diminished binding, $n = 10$). In this group, the CK activity was high, at 2.8 ± 0.1 CK IU/10^8 spermatozoa, indicating a high concentration of sperm cells with cytoplasmic retention. These men should be re-tested, and if the diminished binding is confirmed, these couples may be treated with IUI briefly, and in case of failure with ICSI. Obviously, these application guidelines will need to be confirmed with prospective studies and clinical experience. As with the other biochemical markers, HA binding was largely independent from sperm concentrations. Among men within the <20 × 10^6 sperm/ml concentration range ($n = 18$ of 56 men), three excellent, seven intermediate, and eight diminished HA binders were identified (Huszar *et al.*, 2002).

Male fertility and semen parameters

In considering the potential diagnostic value of the sperm–HA binding assay, it should be noted that the relationship between male fertility and the conventional semen parameters is inconsistent. Indeed, in the two blinded studies of IVF couples, the proportion of normozoospermic men with arrested sperm maturity and failure to cause pregnancies was nine of 22 and 13 of 25 respectively (approximately 45% of the population, Huszar *et al.*, 1992: Ergur *et al.*, 2002).

Contemporary studies directed to the relationship between semen attributes and male infertility focus upon normozoospermic and moderately oligozoospermic men. This is because the IUI experience is similar to that of natural conception, and because the success driven IVF centres will tend to perform ICSI if the semen parameters even remotely suggest potential failure with IVF. There are two major IUI studies that focus upon the relationship between semen parameters and fertility. One is a major collaborative effort conducted at seven sites involving approximately 1600 couples (Guzick *et al.*, 2001). The other work summarizes the data of 26 IUI publications encompassing over 30,000 cycles in 14,000 couples (Ombelet *et al.*, 2003).

The conclusions of both IUI studies indicate that there may be differences in semen attributes between men who are fertile and those who failed to cause pregnancy, but with a substantial overlap. In fact, the authors concluded that assessment of single sperm attributes, even strict sperm morphology, is of limited predictive value.

With respect to IVF/ICSI data, several investigators attempted both IVF and ICSI in the same cycle on sibling oocytes (Nagy *et al.*, 1998; Pisarska *et al.*, 1999; Tournaye *et al.*, 2002: Van der Westerlaken *et al.*, 2006).

In the van der Westerlaken study involving 106 couples (1518 oocytes), 28 of the couples were treated with ICSI, while 78 couples were treated with both IVF and ICSI. Two couples failed to fertilize in both IVF and ICSI, while the 26 women who were only treated with ICSI showed a 57% fertilization rate (182 oocytes). In the remaining 78 patients, 528 oocytes were treated with conventional IVF yielding a 51% fertilization rate, and 858 oocytes were treated with ICSI resulting in a 56% fertilization rate. The pregnancy rates similarly failed to show differences, as the per-transfer rates were 54% for IVF and 48% for ICSI.

Thus, above a threshold motile sperm density, no semen parameters are identified that would predict whether IVF or ICSI is more beneficial for a particular couple. It is anticipated that the sperm–HA binding assay will contribute to an objective assessment of sperm function and male fertility, and thus optimize the mode of fertility treatment. The ability to measure the proportion of spermatozoa that are able to bind to the zona pellucida will greatly enhance the semen analysis with sperm concentration, motility and morphology, and will also improve the epidemiological focus of male infertility classification (Aziz *et al.*, 2006). Indeed, the sperm HA-binding assay is likely to be more on target than the so far most advanced sperm–hemizona assay, as the HA slides are of uniform quality as opposed to the variations of integrity of unfertilized human oocytes.

ICSI sperm selection by HA binding: FISH analysis of spermatozoa in semen and in the respective HA-selected sperm fractions

The increased rate of chromosomal aberrations and other potential consequences of using immature spermatozoa for ICSI are of major concern (Simpson and Lamb, 2001; Van Steirteghem *et al.*, 2002; Barri *et al.*, 2005; Bonduelle *et al.*, 2005). Based on the presence of the HA receptors in mature, but not in immature spermatozoa, coupled with a respective device with an HA-coated surface, it is expected that the method will facilitate the selection of single mature spermatozoa with high DNA integrity and low frequencies of chromosomal aneuploidies for ICSI. As demonstrated (**Figure 6**), the HA-selected mature spermatozoa are devoid of cytoplasmic retention, persistent histones and DNA fragmentation.

Regarding ICSI sperm selection, the efficiency of elimination of aneuploid and diploid spermatozoa from the HA-bound population (Jakab *et al.*, 2005) has been tested in three experiments. The sperm selection studies utilized Falcon Petri dishes (so-called PICSI dishes) that have four marked spots of immobilized HA (Biocoat Inc., Fort Washington, PA, USA).

A drop of washed spermatozoa was placed close to the edge of the HA spot, and the spermatozoa were allowed to migrate spontaneously. The mature spermatozoa that had completed plasma membrane remodelling bound to the HA, while arrested maturity spermatozoa with lower HA receptor concentrations moved freely over the HA (**Figure 6**, left panel). The HA-bound spermatozoa also exhibited vigorous beating with increased tail cross-beat frequency (Huszar *et al.*, 1990b, 2003; Sbracia *et al.*, 1997). After 15 min, the HA-bound spermatozoa were collected with the ICSI micropipette (**Figure 7**, right panel), fixed with methanol–acetic acid and subjected to FISH, using centromeric probes for the X, Y, and 17 chromosomes (Jakab *et al.*, 2005).

In the control semen fraction of 34 men a mean of 4770 spermatozoa were analysed, or 162,210 spermatozoa in total. In the HA bound and micropipette collected sperm fractions, due to the burdens of the task, there were fewer spermatozoa studied. In the first experiment, 7530 spermatozoa (range: 224–1142 per man), and in the second experiment, 9720 spermatozoa (range: 373–1955 per man) were evaluated. In the third experiment of individually selected spermatozoa, 24,420 spermatozoa were evaluated (range: 1086–3973 per man).

In the first experiment, 12 moderately oligozoospermic men (sperm concentration: 20.6 ± 1.7 × 10^6/ml, motility: 52.1 ± 2.5%, all data mean ± SEM) were examined. In the HA-selected spermatozoa versus initial semen with the exception of Y disomy, the frequencies of all other aneuploidies and diploidies declined: 4-fold for 17 disomy, 5.7-fold for sex chromosome disomies, and 6.2-fold for diploidies. Indeed, no matter how high the frequencies were in semen, the HA-selected spermatozoa were within the range of normozoospermic men (**Figure 7**, experiment 1).

In the second experiment (12 normozoospermic patients, sperm concentration:121.3 ± 21.4 × 10^6/ml, motility: 59.5 ± 4.9%), the question of whether HA selection would cause a

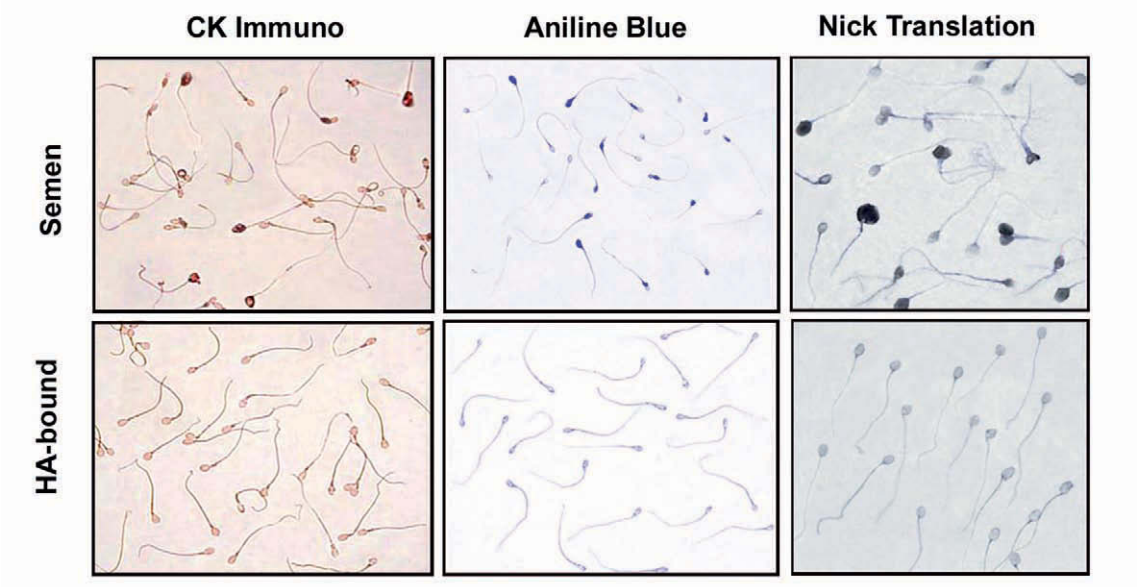

Figure 5. Hyaluronic acid (HA)-bound mature spermatozoa do not contain cytoplasmic retention [creatine kinase (CK) immunostaining], persistent histones (aniline blue staining), or DNA fragmentation (detected by DNA-nick translation; Irvine *et al*., 2000; Sati *et al*., 2004). Upper panels: spermatozoa from whole semen showing various degrees of staining. Lower panels: The HA-bound sperm stained with the respective cytoplasmic and nuclear biochemical markers. HA-bound spermatozoa show clear patterns.

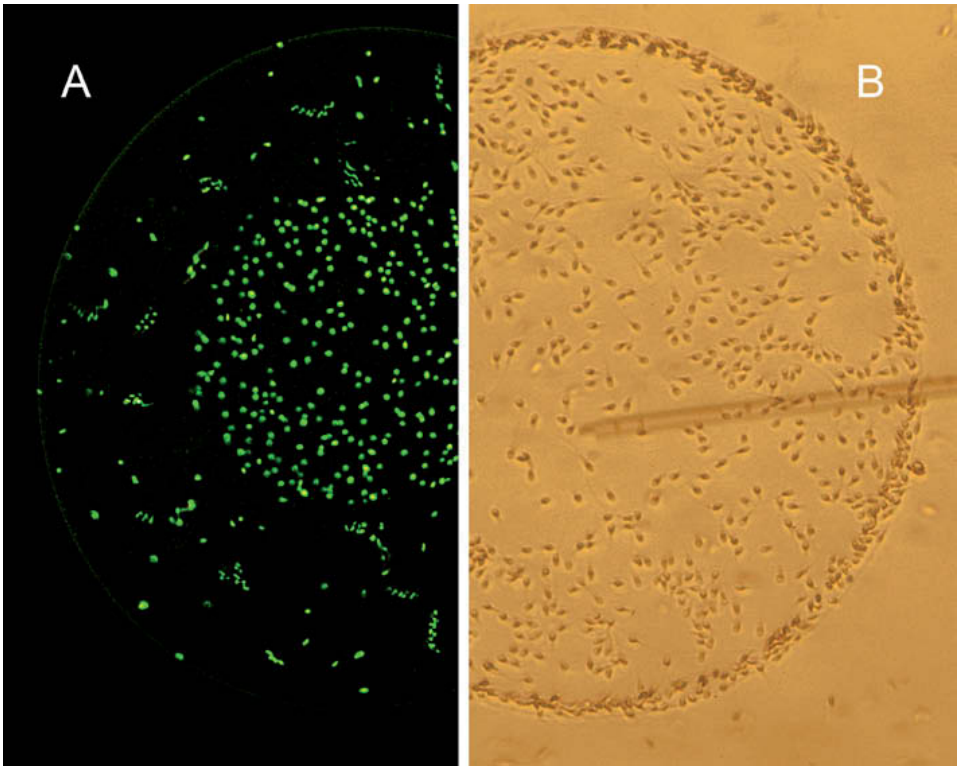

Figure 6. Selection of mature spermatozoa for intracytoplasmic sperm injection (ICSI). (**A**) Time-lapse photography. Spermatozoa applied to the periphery of an HA spot. Mature spermatozoa bind (solid spots) and maintain their tail-beating activity, whereas some arrested maturity spermatozoa, lacking HA receptors, freely proceed over the HA coating. (**B**) After removal of the unbound spermatozoa by gentle rinsing, mature spermatozoa are selected with the ICSI pipette. Reprinted from Jakab *et al*. (2005), with permission from the American Society for Reproductive Medicine.

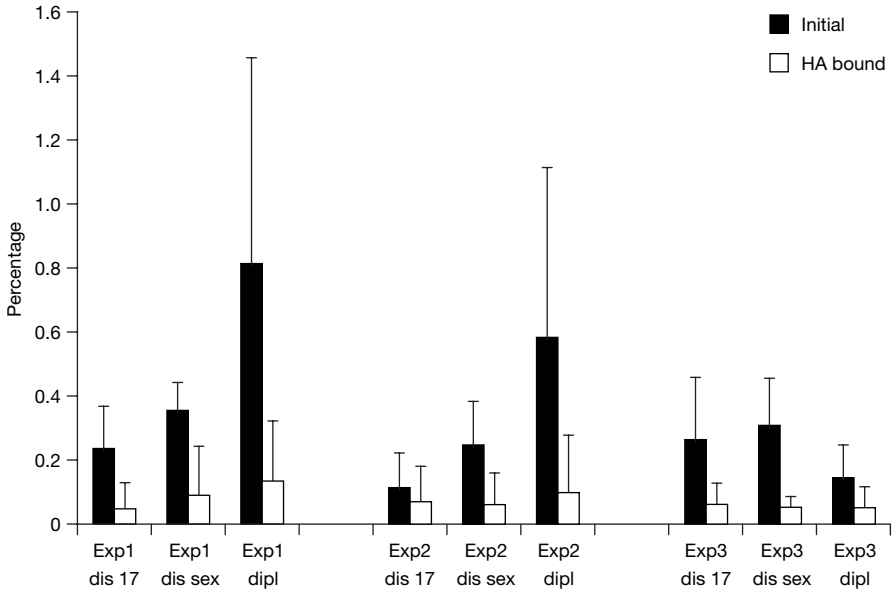

Figure 7. Aneuploidy frequency results after the three experiments (experiments 1, 2 and 3). dis = disomy; dis sex = sex chromosome disomy; dipl = diploidy. Reprinted from Jakab *et al.* (2005), with permission from the American Society for Reproductive Medicine.

decline in chromosomal aberrations even in sperm fractions of highly normozoospermic samples that were further enhanced in mature spermatozoa via gradient centrifugation was addressed. As expected, in the normozoospermic samples, the frequencies of disomies and diploidies were lower compared with those of oligozoospermic men (**Figure 7**, experiment 2). However, HA-binding resulted in a substantial selection effect, as there was a 4.3-fold decline in sex chromosome disomies, and a 5.8-fold reduction in diploidies. As in experiment 1, the frequencies of individual aneuploidies or diploidies were in the very low 0.04–0.1% range.

In the third experiment, 10 oligozoospermic men were studied (sperm concentration: 12.6 ± 1.2 × 10^6/ml, motility: 49.3 ± 4.0%) in a setting similar to IVF laboratories, where the embryologist collects the HA-bound spermatozoa individually. In this approach, which required marathon collection sessions, a mean of 2442 HA-selected spermatozoa were studied in each of the 10 men (range: 1086–3973). As **Figure 7**, experiment 3, indicates, neither the frequencies of disomies, nor the significant reduction in disomy frequencies in the HA-selected spermatozoa, differed from those produced in experiments 1 and 2.

Overall results

In the HA-bound spermatozoa versus unselected spermatozoa, the chromosomal disomy frequencies, with the three probes studied, were reduced to 0.16 from 0.52%, diploidy to 0.09 from 0.51%, and sex chromosome disomy to 0.05 from 0.27% (a 5.4-fold reduction versus a 4- to 5-fold respective increase in ICSI offspring). No matter how high the aneuploidy frequencies in the semen sperm fractions were, the respective frequencies were within the narrow low 0.04–0.10% range per probed chromosome in HA-bound spermatozoa, comparable with the range of normozoospermic fertile men. The 5-fold decline in X, Y and XY disomies is consistent with the increase in chromosomal aberrations in ICSI children conceived with visually selected spermatozoa (Simpson and Lamb, 2001; Bonduelle *et al.*, 2002).

Although the primary ICSI candidates are men with severe oligozoospermia (notwithstanding the fact that success oriented IVF laboratories use ICSI almost exclusively, thus almost any couple may be exposed to the ICSI risks), it was necessary to use oligozoospermic and borderline oligozoospermic samples in order to collect sufficient numbers of spermatozoa to statistically validate the experimental results. However, because ICSI sperm selection reflects the maturity status and binding of single spermatozoon, it is believed that HA-bound mature spermatozoa of a severely oligozospermic man or of men with higher sperm density should not be different. If a spermatozoon binds to HA, it will bind whether there are 10, or thousands of mature or arrested maturity spermatozoa in the drop. Thus, due to the polymorphic nature of spermatozoa, similar to the zona pellucida binding, HA-selection of mature spermatozoa is likely to be independent from seminal sperm concentrations. This hypothesis is well supported by the sperm HA-binding studies, and by the three experiments, in which the aneuploidy frequencies in the HA-selected sperm fractions were similar and within a narrow, low and normal range. The differences between men with more or less mature sperm populations are materialized by the proportion of sperm amenable for HA binding (**Figure 7**).

There are now several laboratories that have initiated the use of HA-mediated sperm selection. It is important that none of the groups that practise the HA sperm selection have reported any adverse effect on fertilization or embryo development. In one 2005 ESHRE presentation, data on 18 pregnancies were reported. Comparing ICSI results with visually selected ICSI spermatozoa (n = 84) versus HA-selected ICSI spermatozoa (n = 18): fertilization rates: 69.7 versus 67.0%; good grade embryos: 56.6 versus 51.7%; pregnancy rates: 45.3 versus 33%; miscarriage rates: 7 versus 0%; take home baby rates: 46.5 versus 39.0% (Sanchez *et al.*, 2005).

In a more recent 2006 study of 26 patients (273 oocytes retrieved and 177 embryos created by ICSI), the fertilization rates with visually selected and HA-selected spermatozoa were similar (66.6 and 61.1%). Among the 22 couples with embryo replacement, eight couples received embryos fertilized with visually selected spermatozoa, seven couples received embryos with HA-selected spermatozoa, and another seven couples received embryos of both origins. The respective pregnancy rates were 25, 57.1 and 57.1% with a significantly higher rates in the two groups that received HA-selected embryos. Regarding miscarriage rates, they were higher in the group that received visually selected embryos (P = 0.013, Worrilow *et al.*, 2006).

Another related 2006 study was performed by the Brussels group on 20 unselected couples treated with ICSI, in which at least eight metaphase II oocytes were recovered. The sibling oocytes were injected either with HA-selected or with visually selected spermatozoa (n = 146 and 145). The fertilization rates (72.9 and 66.9%), oocyte degeneration rates (9.6 and 13.8%), rate of embryo cleavage (cell numbers), embryo quality, embryo transfer and embryo cryopreservation rates were all similar. The authors suggest that in addition to the sperm selection utility in ICSI, the use of HA-selected spermatozoa may be useful in patients undergoing preimplantation genetic screening in which the chromosomal status of the embryos can be related to the sperm selection technique (Janssens *et al.*, 2006).

A relevant publication (Park *et al.*, 2005) reports improvements with HA-selected spermatozoa in porcine IVF. Porcine embryos were produced by IVF, ICSI and ICSI performed with HA-selected spermatozoa. The HA-mediated sperm selection was superior to visual sperm selection in producing chromosomally normal embryos, and increasing ICSI efficiency by reducing the early embryonic mortality and thus enhancing ICSI success rates.

Regarding other means of ICSI sperm selection, it is of interest that there is a new approach based on the electrophoretic separation of spermatozoa (Ainsworth *et al.*, 2005). The data seem to indicate an improvement in DNA integrity within the selected sperm fraction. Because this system is based on electrophoretic charges on the sperm surface, the impact of this selection on sperm maturity and chromosomal aberrations is as yet unclear. Most recently, the group has reported on an ICSI pregnancy with sperm prepared by electrophoretic separation (Ainsworth *et al.*, 2007).

Potential benefits of the HA-mediated sperm selection method for ICSI offspring

HA-mediated ICSI sperm selection, by introducing only mature spermatozoa, will maintain the genetic impact and paternal contribution of sperm to the zygote at the traditional evolutionary level. Thus, the HA method may alleviate the potential problems related to chromosomal aneuploidies and DNA chain fragmentation that presently cause concern due to ICSI fertilization with visually selected spermatozoa that may be of arrested maturity (Huszar and Vigue, 1994; Huszar *et al.*, 2003; Aitken *et al.*, 2003; Cayli *et al.*, 2004; Celic-Ozenci *et al.*, 2004; Sati at al., 2004; Lewis and Aitken, 2005; Seli and Sakkas, 2005; Barri *et al.*, 2005).

The safety of HA-mediated sperm selection is supported by various lines of evidence. First, HA occurs normally in the female reproductive tract and in the cumulus oophorus. Thus, it is likely that HA is carried with the spermatozoa into oocytes even during in-vivo conception. In the case of ICSI, the removal of spermatozoa from HA may cause a few HA molecules to attach to spermatozoa, or cause an extremely small area of the sperm membrane from the region of the acrosomal cap (which is lost otherwise during the acrosomal reaction) to remain attached to the HA.

DNA damage is associated with a decline in pregnancy rates following natural conception, but it has also been linked to diminished success in assisted conception due to reduced rates of fertilization, disrupted development of the zygote and early pregnancy loss (Lopes *et al.*, 1998; Cayli *et al.*, 2003a; Aitken *et al.*, 2004; Seli and Sakkas, 2005; Borini *et al.*, 2006). This is an increasingly important factor because 3–6% of the population in developed countries now utilize assisted conception for reproduction. Another, related, concern is raised by the relationship between paternal and maternal (in-pregnancy) smoking, and other associated oxidative damage to DNA in response to xenobiotics, pesticides, and environmental chemicals which may promote testicular cancer and childhood cancer (Sorahan *et al.*, 1997; Robaire and Hales, 2003; Aitken *et al.*, 2004; Pettersson *et al.*, 2004).

Arrested/diminished maturity sperm with DNA damage and arrested membrane remodelling, that are unable to fertilize in natural conception are likely be eliminated by HA-mediated ICSI sperm selection with potential improvements in various areas:

(i) Following ICSI fertilization with visually selected spermatozoa, there were increased rates of de-novo numerical chromosomal aberrations, and also cytogenetically detectable structural chromosomal aberrations. These are most likely due to increased rates of chromosomal aneuploidies, primarily sex chromosome disomies, in spermatozoa of ICSI fathers (Simpson and Lamb, 2001; Lam *et al.*, 2001; Bonduelle *et al.*, 2002, 2005; Van Steirteghem *et al.*, 2002).

(ii) With visually selected spermatozoa and ICSI, there is a reportedly higher incidence of spontaneous abortions in the 18% range, compared with the 10% rate following normal conception (Van Steirteghem *et al.*, 2002).

(iii) In a recent multi-centre study from five European countries which focused upon 5 year-old singleton children, a potentially increased risk of birth defects was reported; the odds for malformations were 2.77 for ICSI versus naturally conceived children (n = 540 and 538 respectively; Bonduelle *et al.*, 2005).

(iv) Men treated with ICSI also show a higher rate of chromosomal re-arrangements, such as reciprocal and Robertsonian translocations. These rearrangements may be associated with oligozoospermia and infertility, as well as, via interchromosomal effects, dysomies and diploidies. Thus, HA-mediated sperm selection for ICSI may reduce the risk for the chromosomal aberrations for the offspring, if a man has a common origin of chromosomal re-arrangements, numerical aberrations and arrested sperm maturity (Ogawa *et al.*, 2000; Morel *et al.*, 2001; Anton *et al.*, 2004).

(v) Regarding structural chromosomal abnormalities in ICSI-derived pregnancies after visual sperm selection, the incidence of abnormal karyotypes was examined via chorionic villus sampling and amniocentesis. In 1586 subjects, there were 47 children (3%) with abnormal fetal karyotypes, and 25 of these (1.6%) were *de novo*. Regarding the role of sperm maturity, the frequency of structural chromosomal abnormalities was approximately 10-fold higher (24/1120 or 2.1% versus 1/1419 or 0.24%, P = 0.006) following ICSI fertilization by oligozoospermic, and severely oligozoospermic men (Schreurs *et al.*, 2000).

(vi) In line with the idea of the Brussels group who see a potential for fertilization with HA-selected sperm for couples who plan to undergo preimplantation genetic diagnosis or preimplantation genetic haplotyping: Fertilization with HA-selected mature spermatozoa may delineate into the oocyte aneuploidies and chromosomal aberrations found in embryos. Data supporting this approach arising from recent studies (Chatzimeletou *et al.*, 2006; Renwick *et al.*, 2006).

(vii) Using HA-selected mature spermatozoa may increase pregnancy success following ICSI by reduction of sperm derived aneuploidies and defects of oocyte activation that lead to early embryo failure (Baltaci *et al.*, 2006; Menezo, 2006).

Conclusions

The recognition of the HA-binding attributes of mature spermatozoa provides a new approach in andrology testing and in ICSI sperm selection.

(i) The sperm HA-binding test provides a 15-minute microscopic assay for the assessment of the proportion of spermatozoa that would bind to the zona pellucida.

(ii) In spermatozoa selected by the HA-mediated approach, the frequencies of chromosomal disomies and diploidies are within the normal range, independently from the aneuploidy frequencies of the initial semen. Thus, the sperm selection properties of HA are similar to those of the zona pellucida in conventional fertilization. This development addresses a potential major public health concern.

(iii) Mature spermatozoa selected by virtue of HA-binding are also viable, and devoid of persistent histones and apoptosis. HA-selected mature sperm do not exhibit DNA fragmentation. This should improve the paternal contribution of spermatozoa and ICSI efficiency in achieving pregnancies and in reducing early pregnancy loss.

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