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Hyaluronic acid binding by human sperm indicates cellular maturity, viability, and unreacted acrosomal status

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Objective: To test, both in semen and washed-sperm fractions, whether hyaluronic acid (HA) binding is restricted to sperm that have completed cellular maturation.

Design: Comparisons of sperm in semen and in HA-bound sperm fractions.

Setting: University-based diagnostic and research andrology laboratory.

Patient(s): Semen samples originated in men being tested for infertility.

Intervention(s): The attributes of sperm maturity were tested by immunocytochemistry with creatine kinase and HspA2 antisera (highlights cytoplasmic retention in diminished-maturity sperm), aniline blue chromatin staining (detects persistent histones), pisum sativum lectin staining (reveals acrosomal integrity), and the FertiLight viability kit (highlights viable and nonviable sperm).

Result(s): All markers of sperm maturity and immaturity supported the hypothesis that HA-bound sperm are mature. Nonbinding sperm exhibited cytoplasmic and nuclear properties of diminished maturity. The acrosomal status of HA-bound sperm was either unreacted or slightly capacitated, but not acrosome reacted. Only viable sperm exhibited HA binding.

Conclusion(s): Sperm that are able to bind to HA are mature and have completed the spermiogenetic processes of sperm plasma membrane remodeling, cytoplasmic extrusion, and nuclear histone–protamine replacement. Hyaluronic acid–bound sperm show unreacted acrosomes. These studies provide further insights into the relationship between spermiogenesis and sperm function. (Fertil Steril® 2003;79(Suppl 3):1616–24. ©2003 by American Society for Reproductive Medicine.)

Key Words: Human sperm, hyaluronic acid-binding, maturity, HspA2, chromatin integrity, viability, acrosomal integrity

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In the past decade, we have studied various objective biochemical markers of human sperm maturity and function. Measurements of sperm creatine-*N*-phosphotransferase or creatine kinase (CK) indicated that men with low sperm concentrations, who have increased incidences of infertility, also show increased levels of sperm CK activity (1,2). Further studies, using CK enzymatic active-site labeling and CK immunocytochemistry of individual sperm, indicated that the high sperm CK activity is a consequence of increased CK and cytoplasmic protein concentrations. These findings suggested to us that we had identified a defect of sperm development in the last phase of spermiogenesis, when excess cytoplasm is normally extruded and left in the adluminal area as residual bodies (3, 4). Thus, sperm with high CK content, an indication of surplus cytoplasm, have not completed cellular maturation.

Subsequently, we found a second sperm maturation marker, initially thought to be a sperm-specific creatine kinase M-isoform and recently identified as the testis-expressed chaperone protein HspA2 (a member of the HSP70-2 family that, along with the homologous hsc70 [5], is synthesized in two waves of expression [6]). The first wave occurs during meiosis, as HspA2 is part of the synaptonemal complex (7). The second wave of major expression occurs simultaneously with cytoplasmic extrusion, in terminal spermiogenesis.

In three independent studies, we showed close correlations between the decline of sperm

CK activity and increase in HspA2 levels, indicating that the commencement of the second wave of chaperone synthesis and the loss of cytoplasm may be related spermiogenetic events (6, 8, 9). It was also of interest that in blinded clinical studies, the high sperm CK activity and decreased HspA2 levels were consistent with diminished male fertility (10–12). In line with the relationship between the biochemical markers of sperm maturity and spermiogenesis, morphometric attributes of the midpiece and tail were also established that are characteristic for mature and diminished-maturity spermatozoa (13, 14).

In attempting to identify the steps of the fertilization process at which the immature sperm with cytoplasmic retention and diminished HspA2 are deficient, we explored the relationship between zona pellucida–binding competence and maturity by focusing on CK-immunostained sperm samples and their respective sperm–hemizona (halved unfertilized human oocytes) complexes. In the semen samples, there were sperm with various degrees of cytoplasmic retention, as indicated by the CK immunocytochemistry, but all sperm bound to the zona pellucida were clear headed, without detectable staining. Thus, immature sperm with retained cytoplasm, high CK content, and low expression of HspA2 are apparently deficient in the zona-binding site (15).

We hypothesized that, along with cytoplasmic extrusion, a developmentally regulated plasma membrane remodeling also occurs that facilitates the formation of the zona pellucida-binding site. We tested this hypothesis by measuring the concentrations of β1,2,-galactosyltransferase, a marker that is present exclusively on the plasma membrane of sperm. In sperm fractions with various levels of maturity, as detected with the CK and HspA2 levels, there was a very close relationship with β1,2,-galactosyltransferase density. Thus, the data supported that the sperm plasma membrane undergoes a remodeling process during spermiogenesis (16).

Concurrently with the sperm maturation studies, in another line of experiments, we investigated the effects of hyaluronic acid (HA), or hyaluronan, which is a linear repeating polymer of disaccharides, on human sperm function. Hyaluronic acid in the medium increased the velocity and retention of motility and viability in freshly ejaculated, as well as in cryopreserved–thawed human spermatozoa (17, 18). The enhancement of sperm motility and velocity occurred as a direct response to HA, as indicated by two observations: [1] there was an instantaneous increase in sperm velocity and tail cross-beat frequency upon HA exposure and [2] when, after density gradient centrifugation, we transferred the HA-exposed sperm to a standard medium, the motility and velocity properties returned to those of the control sperm. We concluded that HA effects on sperm are likely to be receptor mediated, in line with the evidence established by various laboratories for the presence of the HA receptor in human sperm (19–21).

In the present work, we examined [1] whether mature sperm attach and remain bound to immobilized HA, [2] whether sperm of diminished maturity with low HA receptor expression would exhibit diminished HA binding, and [3] whether HA binding would facilitate the selection of mature sperm via the presence of HA receptors in these sperm. In reporting these studies, we will describe the characteristics of mature HA-bound sperm, including lack of cytoplasmic retention and chromatin maturity. Moreover, we will show that sperm viability is a prerequisite for HA binding and that acrosome-reacted sperm do not bind to HA.

MATERIALS AND METHODS

Design and Methods of the Sperm HA-Binding Experiments

During the course of the sperm–HA binding experiments, we used HA of bacterial origin, which was permanently applied (Biocoat Inc. Fort Washington, PA) to plastic Petri dishes as 100- to 300-μm dots, or to one of the platform surfaces (platform B) of the CellVu glass double-chamber semen counting device (Millennium Sciences, New York, NY). The other platform (platform A) of the CellVu chamber remained unmodified and served for the determination of the motile sperm concentration. To determine the fraction of motile sperm that bound to HA, we counted unbound, motile sperm in the HA-coated chamber and compared them with the total motile sperm in the unmodified chamber: percentage of HA-bound sperm = 100 $\times$ [(motile A $-$ unbound motile B)/motile A]. The semen samples studied (sperm concentration and motility properties of the samples are described at the individual experiments) were leftover portions of semen samples submitted for analysis at the Sperm Physiology Laboratory, Yale University School of Medicine. All studies were approved by the Yale School of Medicine Human Investigation Committee.

Sperm–HA Binding for the Biochemical Studies

For the study of the initial semen sperm and HA-bound sperm, depending on the goals of the experiments, we applied 6- to 8-μL aliquots of the semen samples or washed sperm to glass slides or to the HA-coated surfaces, as described for the individual studies. In the study of the HA-bound sperm fractions, we incubated a semen drop layered on the HA surface for 15 minutes at room temperature. After that we applied a gentle rinsing step with human tubal fluid medium (HTF; Irvine Scientific, Irvine, CA), containing 0.5% BSA, to remove the unbound sperm. The HA-bound sperm were then observed in the wet mount or were air dried and fixed with methanol acetic acid, for aniline blue staining, or with formaldehyde, for immunocytochemistry. The HA-coated surfaces allowed the observation of sperm bound to HA, whether by light, phase-contrast, or fluorescence microscopy.

The HA-bound sperm were subjected to immunocytochemistry with CK, to chromatin staining with aniline blue, or to viability or acrosomal integrity evaluation with the respective reagents. In experiments controlling for sperm binding to the HA-coated surfaces, we used glass slides with components of the HA attachment process but without HA. These control surfaces did not exhibit sperm binding.

For sperm washing, semen was diluted with three volumes of HTF containing 0.5% BSA, and the mixture was processed by centrifuge at 600 $\times$ *g* for 15 minutes at room temperature. The sperm pellet was resuspended in HTF for the experiments.

Creatine Kinase Immunostaining of Spermatozoa

The procedures used were described elsewhere (4, 15). Both initial semen and HA-bound sperm fractions to be studied were fixed with 3.7% paraformaldehyde in phosphate buffer/sucrose (PB-suc) for 20 minutes at room temperature. After removal of the formalin, the slide was allowed to air dry. After three washing steps with PB-suc, the spermatozoa were exposed to a 3% bovine serum albumin blocking solution in PB-suc at room temperature. After further washing with PB-suc, the sperm were exposed to a 1:1000 dilution of polyclonal anti-CK-B antiserum (Chemicon Co., Temecula, CA) or to 1:1000 dilution of the HspA2 antiserum generated in our laboratory (6). Subsequent to more PB-suc washes, the slide was processed with a biotinylated second antibody at a 1/1000 dilution. After this step, the slides were exposed to a Vector horseradish peroxidase/ABC kit (Vector, Burlingame CA) according to manufacturer's instructions. ABC treated slides were further processed with diaminobenzidine (DAB) and hydrogen peroxide (Sigma, St. Louis MO). The resulting brown color on the ABC-treated slides highlighted areas of cytoplasmic retention. The specificity of the staining was established by using preimmune serum in place of the first antibody, or by applying the second antibody only.

Aniline Blue Staining of Sperm Chromatin

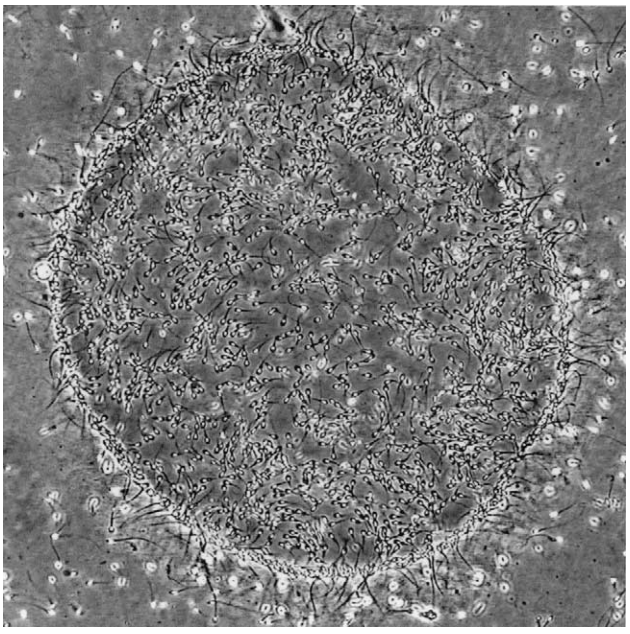
Sperm smears were dried on glass slides and stained with a 5% aniline blue solution acidified to about pH 3.5 using acetic acid. The slides were washed and air dried before evaluation. Sperm with immature chromatin due to persistent histones are darkly stained (22–24).

FertiLight Sperm Viability Test

This test was carried out according to the manufacturer's instructions (Molecular Probes, Eugene, OR). We added 0.5 μL of component A (1:50 dilution of SYBR, a small molecular weight DNA dye that is capable of penetrating membranes of viable sperm), to 100 μL semen and incubated for 5 minutes at 37°C. Subsequently, 0.5 μL of component B (undiluted propidium iodine solution, a red DNA stain incorporated only by nonviable sperm) was added, and the sperm were incubated for a further 5 minutes. This was followed by assessment of the proportion of viable (green fluorescence) and nonviable (red fluorescence) sperm under the fluorescence microscope. The small proportion of sperm (<5%) with declining viability, which stained yellow, were not counted as either viable or nonviable.

FIGURE 1

Phase contrast microscopy of sperm in semen binding to an HA spot. Please observe the sperm bound to the perimeter of the HA with a head first orientation and the sperm tails pointing away from the HA. Some of the sperm in the field did not bind to the HA.



Huszar. Hyaluronic acid-bound human sperm. Fertil Steril 2003.

Acrosomal Status of HA-Bound Sperm

Washed sperm (7–10 μL, 30 $\times$ 10^6 sperm/mL) after 4 hours of incubation in capacitating medium (human tubal fluid with 0.5% albumin, 5 mM $CaCl_2$, and 3μM progesterone at 36°C in an 5% CO_2 incubator) were spread with the pipette tip on the HA-coated slides. The slide was then placed in a humidity chamber for 15 minutes at room temperature and then washed gently and repeatedly with phosphate-buffered saline until all moving or floating sperm was removed. Another aliquot of the preincubated sperm solution (control) was placed on a clean glass slide. The slides were air dried and fixed with 95% ethanol, then dried again. A drop of Pisum sativum lectin solution (100 μg/mL in distilled water; fluorescein isothiocyanate conjugate labeled; Sigma, St. Louis MO) was placed over the sperm spots, and the slides were placed in a dark humidity chamber for 15 minutes at room temperature. The slides were then washed three times with phosphate-buffered saline, fixed with paraformaldehyde, mounted with anti-fade medium, and ob-

FIGURE 2

Time-related binding of sperm to HA in 15 semen samples (mean ± SEM). The percentage of HA-bound sperm was established by videotaping the process for 2-minute periods up to 22 minutes. Maximum binding occurred by the 8–10 minute period.

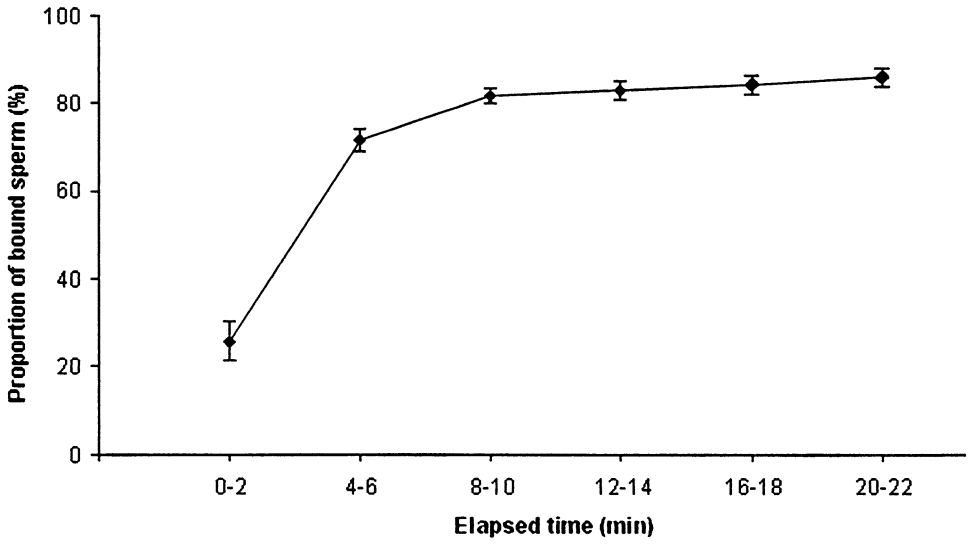


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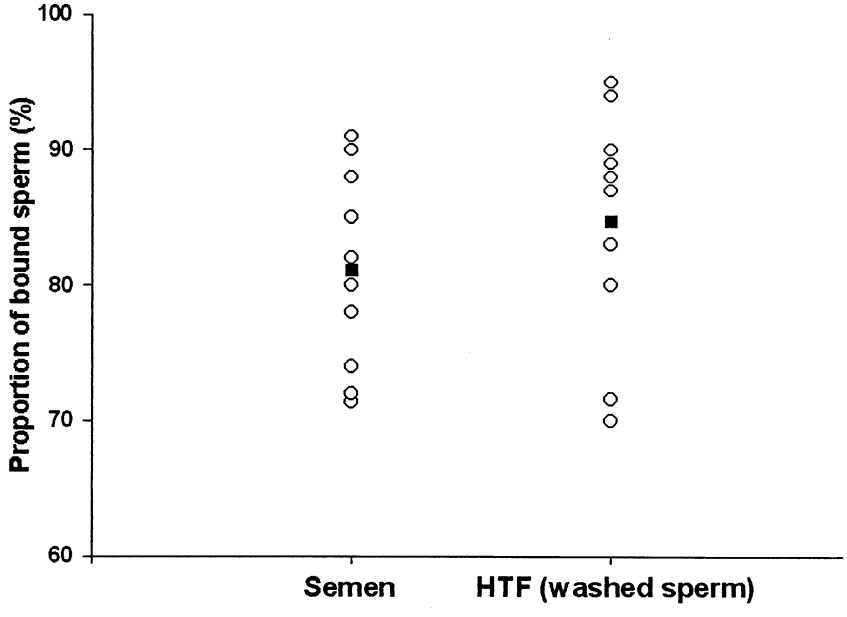
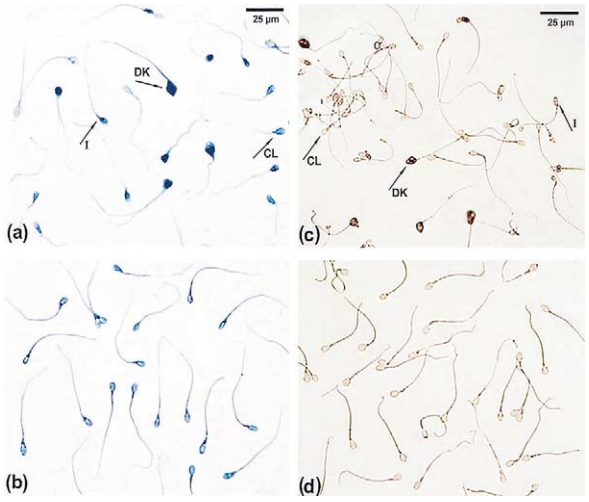
FIGURE 3

Hyaluronic acid binding by sperm in semen and by washed sperm after a 10-minute incubation period (○, 10 individual patients; ■, mean; N = 10 men studied).



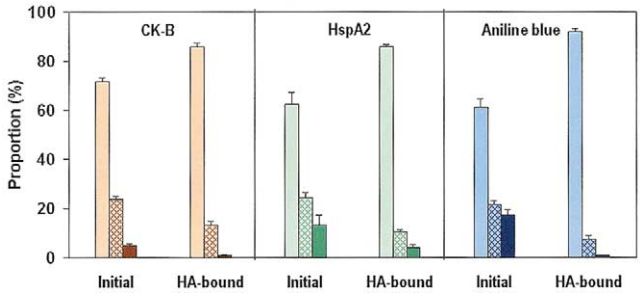
Huszar. Hyaluronic acid-bound human sperm. Fertil Steril 2003.

FIGURE 4



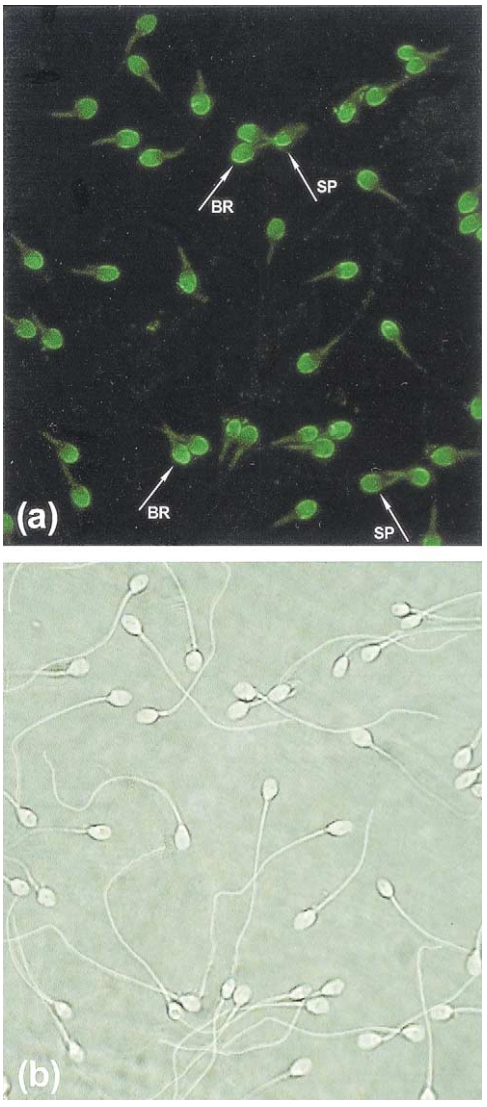
Huszar. Hyaluronic acid-bound human sperm. Fertil Steril 2003.

FIGURE 5



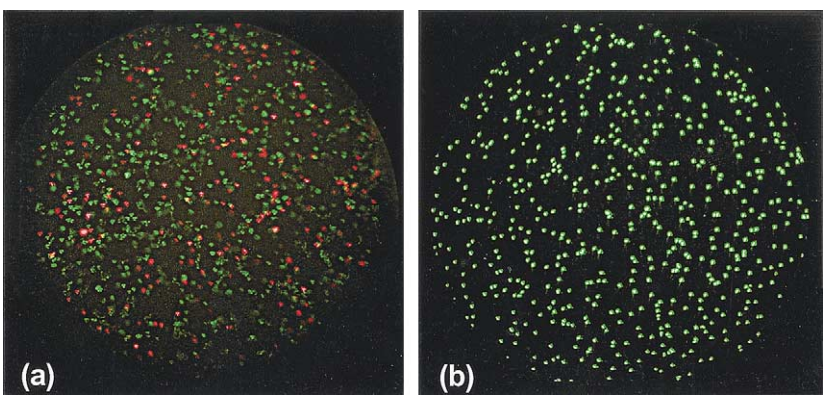
Huszar. Hyaluronic acid-bound human sperm. Fertil Steril 2003.

FIGURE 7



Huszar. Hyaluronic acid-bound human sperm. Fertil Steril 2003.

FIGURE 6



Huszar. Hyaluronic acid-bound human sperm. Fertil Steril 2003.

served under a fluorescence microscope at 494 nm excitation, 520 nm emission (25).

Photography and Imaging Methods

Photographic images of various sperm preparations were captured via an Olympus Camedia Zoom C-4040 digital camera employing a bracketing technique and uploaded via Olympus Camedia Master 2.5 software (Olympus Optical Company, LTD, Tokyo, Japan). Underexposure was used for fluorescent signals. The images were sized and processed for printing using the Adobe Photoshop 5.0 program (Adobe Systems, Inc, San Jose, CA)

Statistical Analysis

All the values were expressed as mean ± SEM. To compare the various groups and to make the comparisons, we used Student's *t* test, and we used Mann-Whitney U test via the computer-based statistical programs SigmaStat and SPSS. Statistical significance was set at $P<.05$.

RESULTS

Sperm Binding to HA

To explore whether the binding of sperm to HA is sufficiently firm to form a permanent attachment, sperm fractions were applied to the HA-coated surfaces. Indeed, sperm did attach to the HA. Binding occurred uniformly via the heads of sperm. There was also a sperm population surrounding the HA spot that did not bind (Fig. 1).

The time-related binding of sperm was examined in the CellVu double chamber glass slides. Identical aliquots of the samples were added to both the uncoated (A side) and to the HA-coated (B side) chambers in 15 men with good HA-binding properties (sperm concentration, 92.0 ± 16.0 million per milliliter; motility, 64.0% ± 4.0%). The motile and motile HA-bound sperm in the A and B chamber, respectively, were assessed by video recording at various time points and were expressed as percentage HA bound according to the formula described in Materials and Methods. As Figure 2 indicates, maximum binding occurred within an 8–10 minute period, and with a fairly narrow sample-to-sample variation. The proportion of sperm bound to HA was not different whether the sperm were applied in semen or after washing and re-suspending in HTF medium (sperm binding in semen: 81.1% ± 2.3%; range: 71%–91%); sperm binding in HTF: 84.7% ± 2.7% (range: 70%–95%; Fig. 3).

We observed that not all sperm were able to bind and that the proportion of HA-bound sperm and those that remained unattached showed a man-to-man variation. In addition to the sperm populations that did or did not bind, there was a small proportion of weakly binding sperm (<5%) that bound briefly to the HA, detached, and bound briefly again at a different site. The weakly binding sperm probably represent a population with low density of HA receptors.

Sperm Maturity and HA Binding

The primary visual characteristic that distinguishes mature sperm from sperm of diminished maturity is the absence or presence of cytoplasmic retention after immunostaining for a cytoplasmic protein. We have demonstrated previously that only mature sperm with no cytoplasmic retention bind to the zona pellucida (15). In the present studies, we examined the relationship between sperm maturity and HA binding by using an experimental design similar to that used for the 1994 sperm-hemizona experiments. Initial sperm samples and their respective HA-bound sperm fractions were fixed with formaldehyde and subjected to immunostaining with a CK-B antiserum, to highlight cytoplasmic retention in the sperm head (30 samples studied; sperm concentration, 39.7 ± 8.9 million per mL; motility, 54.0% ± 2.4%). There were three populations: clear (mature), lightly stained sperm (intermediate), and darkly stained sperm (diminished maturity).

The initial fractions show higher proportions of intermediate and darkly stained sperm having different degrees of cytoplasmic retention. However, as with the hemizona (15), the HA-bound sperm consisted of mature sperm only, with no cytoplasmic retention and with symmetrical oval heads (Fig. 4A and B). The bar graph of Figure 5 (CK-B) provides a quantitative treatment of the binding pattern (200 sperm evaluated in the semen and HA-bound fractions, respectively; 12,000 sperm in all; $P<.001$ in all comparisons, n = 30 men). All slide evaluations were carried out in a blinded manner using two or three different investigators.

Figure 4. (*Left*) CK-B immunocytochemistry of the initial semen and HA-bound sperm fractions for visualization of clear mature sperm, and sperm with various degrees of cytoplasmic retention. (**a**) Sperm originating in the initial semen fraction, (**b**) HA-bound sperm. The CL, I, and DK letters designate clear (mature), intermediate, and dark (diminished maturity) sperm. (*Right*) Aniline blue staining of sperm in the initial semen and HA-bound sperm fraction for detection of spermatozoa with various levels of persistent histone content. (**c**) Sperm originating in the initial semen. (**d**) HA-bound sperm.

Figure 5. The proportion of mature (□ clear), intermediate (▦), and immature (■ dark) sperm in the semen and HA-bound fractions. Brown bars: CK-B immunocytochemistry; Green bars: HspA2 immunocytochemistry; Blue bars: aniline blue stain.

Figure 6. Sperm viability and HA binding. Sperm treated with the FertiLight kit were applied to HA-coated slides, and after 15 minutes the slides were gently rinsed. (**a**) Semen sperm fraction. (**b**) Hyaluronic acid–bound sperm fraction after the rinsing step.

Figure 7. Hyaluronic acid–bound sperm fraction treated with fluorescein isothiocyanate conjugate–labeled lectin. (**a**) The fluorescence microscopy image indicates that all HA-bound sperm exhibit either bright (**BR**) or intact slightly patchy (**SP**) capacitating acrosomal membrane patterns. Sperm in which the capacitation pattern was more advanced did not bind to the HA-coated surfaces. (**b**) In the light microscopy picture of the same field, each sperm head is well identifiable.

In a further study focusing on the relationship between sperm maturity and HA binding, with the other maturity marker, HspA2 staining, we reconfirmed the differences in the proportions of mature (clear), intermediate (lightly stained), and immature sperm (dark staining) in semen and in the HA-bound fractions (5 men studied; sperm concentration, 46.0 ± 13.7 million per milliliter; motility, 55% ± 5.9%). An enrichment in mature sperm and a decline in the proportion of immature sperm occurred in the HA-bound fractions (Fig. 4 HspA2, 300 sperm evaluated in each fraction, 3,000 sperm in all, all comparisons $P < .01$– $< .001$, N = 5 men).

Structure of Sperm Nucleoproteins in the HA-Bound Spermatozoa

The stain aniline blue selectively highlights persistent histones in the nuclei of immature sperm, whereas mature sperm in which histones have been replaced with protamines remain clear after the staining step. The aniline blue staining patterns of clear mature and aniline blue–stained intermediate and dark sperm are shown in Figures 4C and D. To further study the apparent relationship between the cytoplasmic and nuclear aspects of sperm maturity/immaturity and the selectivity of sperm binding to HA, we extended the HA-binding experiments described in Figure 4 to aniline blue–stained sperm originating in semen and in HA-bound sperm fractions (sperm concentration: 44.6 ± 8.4 million sperm/mL; motility: 61% ± 5.8%, N = 5 men). In agreement with the CK-B immunostaining results, the semen fractions contained sperm with mature (clear), and intermediate and immature (dark) aniline blue staining. Also, in agreement with the CK-B immunostaining data, the proportions of clear mature sperm increased in the HA-bound fractions. Thus, sperm with lesser maturity were deficient in binding (Fig. 5: aniline blue; in each semen and HA-bound fraction, 200 sperm were evaluated, 2,000 sperm in all; all comparisons, $P < .001$). Thus there is a uniform pattern in the occurrence of sperm types and in the proportion of the types in the semen and HA-bound fractions, whether one uses the CK-B and HspA2 cytoplasmic markers or the aniline blue nucleoprotein probe.

Sperm Viability is a Prerequisite for HA-Binding Ability

Because HA binding is receptor mediated, we examined whether sperm viability and consequential membrane integrity, as detected by the FertiLight viability kit, would affect sperm binding. We found this to be the case in two lines of experiments. First, after mild denaturing conditions, such as exposure to heat at 45°C or 5% ethanol, which diminish the proportion of viable sperm, sperm binding to HA was lost. Further, in the sperm–HA binding experiments, only the viable green-stained sperm, and none of the red (nonviable) sperm, remained bound after gentle washing (Fig. 6).

Acrosomal Integrity and HA Binding

The maintenance of acrosomal integrity in the HA-bound sperm fraction was studied by the binding pattern of fluorescence-labeled lectin. Three patterns were distinguishable (27): [1] even distribution of fluorescence over the sperm head (intact acrosome, Fig. 7); [2] patchy fluorescence and/or meridional linear fluorescence around the sperm head (capacitating spermatozoa); [3] dark sperm head without fluorescence (acrosome-reacted dull sperm).

We studied five samples (sperm concentration, 78.8 ± 9.6×10^6/mL; motility, 65.3% ± 3.2%; preincubation acrosomal status, intact: 86.2 ± 2.9%, capacitated: 9.1 ± 0.4%, acrosome reacted: 4.3 ± 0.2%) after the capacitation period as described in Materials and Methods. The proportions of intact, capacitated, and acrosome-reacted sperm in the control and HA-bound fractions were as follows: intact: 69.2% ± 5.3% and 89.5% ± 3.6%, capacitating: 18.4% ± 6.3 and 10.2% ± 3.5%, acrosome reacted: 8.5% ± 0.9 and 2.3% ± 0.1% (N = 300 sperm evaluated in each control and HA-bound sample, 3,000 sperm in all, $P < .05$–$< .001$). Thus, the data indicate that sperm with intact acrosomes may preferentially bind to HA and that some capacitating sperm also bind; however, acrosome-reacted sperm do not bind to HA.

DISCUSSION

In the past 15 years, we have studied several key events of sperm maturation, including cytoplasmic extrusion and expression of the HspA2 chaperone protein (1, 4, 6, 9–11, 15). In experiments related to sperm function and fertilizing potential, we have established that, simultaneously with cytoplasmic extrusion in spermiogenesis, there is a remodeling of the plasma membrane that facilitates the formation of the zona pellucida– and HA-binding sites (16). The present studies with immobilized HA showed that sperm firmly bind to HA. However, similar to the response of HA-mediated increase in velocity, not all sperm exhibited HA-binding ability (17, 18). We thus formulated the hypothesis that HA binding is related to sperm maturity. In the present work, we have examined the validity of this hypothesis and have also studied the properties of both HA-binding and HA-nonbinding sperm using various biochemical markers of sperm maturity and function.

The data of the various markers studied indeed supported the idea that HA binding is related to sperm maturity. The time curve of HA binding by mature sperm was consistent among the samples of various sperm concentration or motility or proportion of HA-binding mature sperm. The extent of binding was similar, whether sperm were in semen or in HTF medium, indicating that binding is associated with the presence of the HA receptors on the sperm surface. We also detected a man-to-man variation in sperm HA binding. This finding is in agreement with the earlier reported variation in CK and HspA2 levels, which are due to differences in the proportion of mature and diminished-maturity sperm among

semen samples (2, 4, 9–11, 26, 27). All sperm were bound to HA via their heads. This binding pattern has also been described in monkey sperm, in which HA binds to the PH-20 protein, which is exclusively distributed throughout the plasma membrane of the sperm head (28, 29).

In line with earlier findings, sperm bound to HA exhibited a uniform shape identical to that of the mature sperm and to sperm bound to the hemizona (14, 15). Immature sperm did not bind to HA and was stained dark with either of the CK-B or HspA2 cytoplasmic retention protein markers. In addition to cytoplasmic aspects, we have also investigated chromatin structure in the sperm nucleus via aniline blue staining, which detects the persistence of histones that are not replaced with protamines, as normally occurs in ejaculated sperm. The incidence of aniline blue–stained sperm has been shown to correlate with abnormal sperm morphology, delayed decondensation of DNA, and diminished male fertility (24–26). In the initial semen fraction, reflecting the diversity of sperm maturation, there are sperm with various degrees of aniline blue staining, whereas in the HA-bound fraction, almost exclusively mature sperm with clear heads are observed (Fig. 4). The distribution of mature and immature sperm, either by cytoplasmic retention or by chromatin maturity, show similar patterns in the initial semen and HA-bound sperm fractions, providing further evidence for common spermiogenetic regulation (Fig. 5).

The common pattern among the nuclear and cytoplasmic attributes of sperm immaturity is likely due to the diminished levels of the homologous 70-kDa chaperones, HspA2 and Hsc70 in immature sperm, and to the consequential lack of chaperone assistance in transport of various proteins such as DNA repair enzymes, protamines, and structural proteins needed for sperm remodeling (6, 30, 31).

The experimental design that combined immunocytochemistry with aniline blue staining and HA binding provided three lines of evidence: [1] extent of cytoplasmic retention and aniline blue staining in individual sperm are related to each other and also to HA-binding ability. Hyaluronic acid–bound sperm show no cytoplasmic retention or aniline blue staining. Thus, HA binding selects the mature sperm population. [2] Sperm binding to HA occurs via the plasma membrane HA receptors. Acrosome-reacted sperm lose HA-binding ability. [3] Nonviable sperm have lost their HA-binding capacity. This is most likely due to diminished membrane integrity and HA receptor stability in nonviable sperm (17, 18).

In summary, we report that sperm binding to immobilized HA allows identification and separation of sperm that are able to form an attachment. Using objective biochemical and morphometrical parameters, we explored the maturity characteristics of sperm that are bound or fail to bind to HA. Bound sperm show no cytoplasmic retention and contain mature-type chromatin. Further, we have demonstrated that sperm with HA-binding ability are viable and that the acrosomal status is either intact or slightly capacitated. All these data support the concept that sperm binding to HA via the plasma membrane HA receptors represents unequivocal evidence of completed spermiogenetic maturation.

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