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In Vitro Sperm-Binding Assay to Distinguish Differences in Populations of Human Sperm or Damage to Sperm Resulting From Cryopreservation

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ABSTRACT: Annually, >1.3 million men are members of couples seeking help because of infertility. Semen from many of these men contains reasonable numbers of motile and normal sperm, but for a subset of individuals, many sperm are deficient in ability to bind to the zona pellucida during *in vitro* fertilization. Diagnosis of this defect has been hampered by lack of a low-cost test. Molecular similarity exists between the perivitelline membrane of a hen's egg and the mammalian zona pellucida. These facts and some preliminary data led to evaluation of binding of human sperm during incubation for 60 minutes at 37°C to an extract of chicken perivitelline membrane coated in microwell assay plates. The sperm-binding assay had inter- and intraassay plate variations of 21 and 12%, respectively, using washed fresh sperm. All seminal samples were normal, except a few that had 36 to 50% motile sperm with a low rate of sperm movement (if there is a low rate of movement, World Health Organization [WHO] criterion for normalcy is >50% motile). Nevertheless, this sperm-binding assay detected differences among individuals in percentage of sperm bound. Based on data for two to four ejaculates from each of eight occasional sperm donors, the coefficient of variation for ejaculates within donor averaged 31%, and means for the donors differed ($P < 0.02$). Percentage of sperm bound ranged from <1 to 38% for fresh semen from 57 men and from <1 to 13% for frozen–thawed semen from 34 men. Percentage of motile sperm accounted for <30% of the variation in percentage of sperm bound. In a direct comparison based on 17 ejaculates, aliquots evaluated fresh averaged 13% sperm bound, versus 2% for frozen–thawed aliquots. We concluded that the egg membrane substrate used in these microwell assay plates might serve as the basis for a diagnostic assay. However, it remains to be established whether samples of human semen with a low percentage of sperm binding indeed have relatively low fertilizing potential.

Key words: Sperm quality, fresh versus frozen semen.
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The initial event in fertilization is binding of one or more sperm to the egg investments. This involves a multiplicity of ligands on the sperm surface that interact synergistically and sequentially with egg investments to first bind the sperm to the egg and then collectively impart species specificity (Yanagimachi, 1994; Töpfer-Petersen and Calvete, 1995; Töpfer-Petersen et al, 1995). Obviously, failure of initial binding blocks subsequent steps. On the basis of data from hemizona pellucida assays and results in *in vitro* fertilization (IVF), sperm from ≳15% of males presenting at infertility clinics demonstrate essentially complete failure to bind to the zona pellucida (Coddington et al, 1994; Oehninger et al, 1997; Oehninger, personal communication). In a small, prospective study of IVF/gamete intrafallopian transfer couples, the sperm of 17% of men did not bind to the zona pellucidae of their partner's oocytes, although donor sperm bound to these oocytes (Windt et al, 1996). Sperm samples demonstrating <65% fertilized oocytes in IVF also had low binding in the hemizona assay (Oehninger, 1992). In part, failure of sperm to bind in a hemizona assay was associated with teratozoospermia and especially with immaturity of sperm (Huszar et al, 1994). However, even for populations of human sperm selected by swim-up from semen provided by normospermic patients, the coefficient of variation (CV) for number of sperm bound in a hemizona assay was 68% (Franken et al, 1993). Hence, it is likely that mature and morphologically normal sperm also differ in ability to bind to the zona pellucida. A simple diagnostic assay for sperm–egg binding, not requiring scarce human zona pellucidae, might have value in patient management.

In mammals, the zona pellucida is comprised of three glycoproteins, ZP_1, ZP_2, and ZP_3, and these have a pivotal role in fertilization (Yanagimachi, 1994). Loose nonspecific binding of a motile spermatozoon to the zona is followed by strong attachment to receptors on ZP_3 and initiation of a cascade of events that culminates in a sper-

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matozoon entering the oocyte. The analogous structure in poultry is the inner lamina perivitelline, in which the core region, "cZP," has 57% amino acid identity with the core human hZP_3 sequence (Waclawek et al, 1998) and probably has higher homology in epitopes actually involved in binding sperm. Given the structural and functional similarities between the inner lamina perivitelline and the zona pellucida, it is not surprising that the former membrane can bind sperm from several species and provide the basis of an assay for subfertile males (Barbato et al, 1998; Amann et al, 1999a; Barbato, 1999). This report shows that inter- and intraplate variations with this assay are appropriately low, that semen from individual normospermic men differs in percentage of sperm bound to the membrane substrate, and that cryopreservation reduces the percentage of sperm bound.

Materials and Methods

The buffer used to wash sperm and perform the binding assay was modified human tubal fluid (mHTF; Irvine Scientific, Santa Ana, California). It was prepared with 5 mg/ml polyvinyl alcohol (P-8136; Sigma Chemical Co., St. Louis, Missouri) rather than with serum albumin.

Semen was made available by California Cryobank Inc (site 1), the Penn State Geisinger Health System (site 2), and Yale University School of Medicine (site 3), consistent with local institutional review board approvals. At site 1, semen was evaluated from 19 individuals being screened to determine whether seminal quality and resistance of sperm to freezing and subsequent thawing was sufficient for them to be designated as commercial donors; they are termed prospective donors. At site 2, data were available for 16 seminal samples from male partners in couples being evaluated for infertility, although all men were essentially normospermic. Twelve samples met World Health Organization (WHO) criteria (World Health Organization, 1993), and semen from four males was marginal because of 45 to 50% motile sperm with a low rate of movement. At site 3, the 41 ejaculates were from 10 occasional sperm donors (one to four ejaculates each) and from 12 male partners of couples being evaluated for infertility (one ejaculate each). These male partners also were essentially normospermic, although semen from three had only 36 to 45% motile sperm. Because most of the male partners at sites 2 and 3 were normal, hereafter they are termed subjects rather than patients; those whose semen did not meet WHO criteria are identified in Figure 1.

After liquefaction, aliquots of semen were washed once (at site 1 or site 3) or twice (site 2) by dilution with mHTF and centrifugation (10 to 15 minutes at ~350 × *g*), and then sperm were resuspended to a desired concentration. In one study at site 1, each ejaculate was split after liquefaction, and one aliquot was used fresh for evaluations of percentage of sperm bound, while remaining semen was processed for cryopreservation by the procedure routinely used (addition of glycerol-containing buffer, making the suspension ~5% (v/v) glycerol), dispensed into one to three plastic vials, frozen to −196°C, and shipped at −196°C to State College, Pennsylvania for evaluation. Cryopreserved semen was thawed (for 10 minutes in 37°C water), gradually diluted to 15 ml (with eight additions of mHTF, each reducing glycerol concentration by ~10%), processed by centrifuge, and resuspended in mHTF to the desired concentration.

The BioPore sperm-binding assay (SBA) used to quantify sperm–egg binding was a proprietary version of assay #3 described by Barbato et al (1998), as described in detail by Amann et al (1999a). The substrate within the microwells was a protein extract prepared from chicken egg membranes. Before use, wells in the SBA plates were hydrated by washing twice with mHTF. For data reported herein, sperm suspensions were prepared at 5 × 10^6 sperm/ml (on the basis of dose–response data in Amann et al, 1999a), 100-μl aliquots were dispensed into each of six microwells in a SBA plate, and the plate was incubated for 60 minutes at 37°C. For certain samples, duplicate aliquots were prepared independently (after centrifugation) and were assayed in the same SBA plate or in duplicate plates. The residual sperm suspension was evaluated for percentage of motile sperm before and after similar incubation. The motility and binding data were used to calculate the coefficient of determination (r^2), namely, the proportion of the variation in percentage of sperm bound explained by percentage of motile sperm, from the correlation coefficient (*r*; Knapp and Miller, 1992). If *r* is significant, r^2 also is significant, even though one attribute may account for <25% of the variation in the other measurement. This approach cannot define cause and effect, but we assumed that good sperm motion facilitates sperm binding, not vice versa.

After a 60-minute incubation, unbound sperm were decanted from the SBA plates, all wells were washed three times with mHTF to remove unbound sperm, and the plates were dried. The DNA in each well, from sperm or standards, was stained with YoPro-1 (Molecular Probes, Eugene, Oregon), and fluorescence was quantified with a fluorometric microwell plate reader (Barbato et al, 1998). Percentage of sperm bound in each microwell was calculated, assuming 2.5 pg DNA per spermatozoon (Amann et al, 1999a). Data for six microwells were averaged to obtain mean percentage of sperm bound for that sample or replicate. Earlier studies (Barbato et al, 1998; Amann et al, 1999a) had revealed that sperm rendered immotile by ATP depletion (blocking the ATP regeneration system) or snap-freezing did not bind (<0.01%) to the wells of an SBA plate.

Precision of the SBA was evaluated in terms of standard criteria: interplate and intraplate variations. Interplate variation was evaluated using data for 11 ejaculates, for which the same sperm suspensions were evaluated using duplicate SBA plates. The difference (regardless of sign) between the two values of a pair averaged 21%. Intraplate variation was evaluated using data for 35 ejaculates (representing all sites), for which two aliquots of stock suspension (after centrifugation) were independently diluted to 5 × 10^6 sperm/ml and were dispensed into different sets of six wells on the same SBA plate. For a given set of six wells, the among-well CV averaged 18% (n = 70). For the 35 ejaculates, mean values ranged from 3 to 36% sperm bound. There was no significant difference between the two values for a pair (paired *t*-test), the paired values were highly correlated (r = 0.93) with each other, and the average difference (regardless of sign) in percentage of sperm bound between the two six-well averages was 12% of the respective mean.

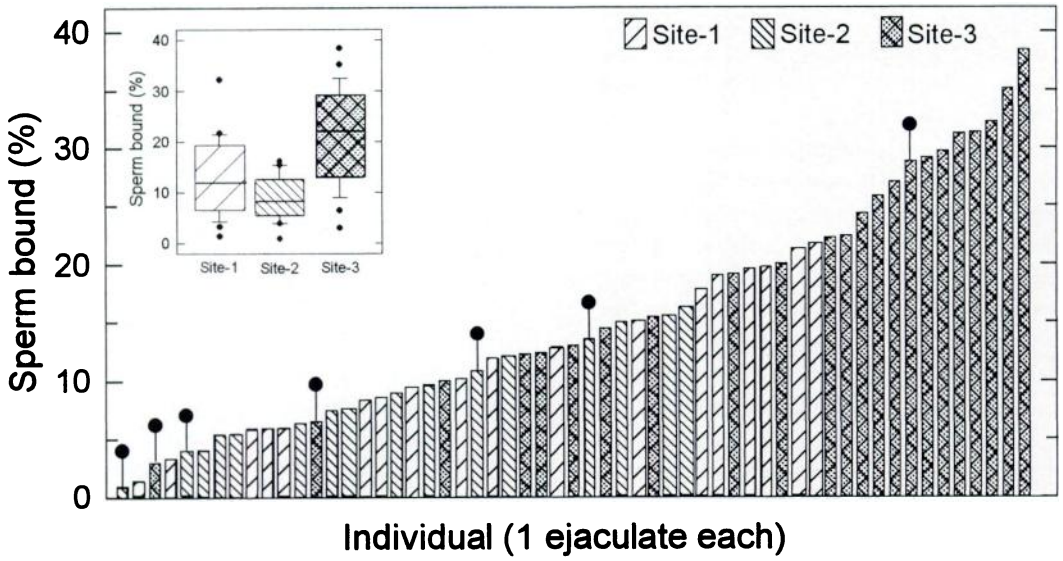

FIG. 1. Range in percentage of sperm bound in SBA for fresh semen from 57 men, each evaluated at one of three geographic sites. For men providing two to four ejaculates, only data for the first ejaculate are included in this summary. Data for seven samples with 36–50% motile sperm with a low rate of movement are designated (ball flag). As evident from the insert, site affected percentage of sperm bound ($P < 0.01$; the box plots show the 25th and 75th percentiles and median, together with whiskers at the 10th and 90th percentiles and outliers).

Results

Binding of Sperm in Fresh Semen

Fresh semen was evaluated at all three sites. At site 1, semen from 19 prospective donors was evaluated, and values ranged from 1 to 32% sperm bound (insert, Fig. 1). At site 2, values for 16 seminal samples from subjects ranged from <1 to 16% sperm bound. At site 3, values for 29 ejaculates from 10 occasional donors ranged from 6 to 38% sperm bound, and those from 12 subjects ranged from 3 to 35% sperm bound. For the seven subjects at sites 2 or 3 whose semen (one sample each) contained a slightly lower percentage of motile sperm, values ranged from 1 to 29% bound. For all 76 ejaculates evaluated, values ranged from <1 to 38% sperm bound.

Percentage of sperm bound was essentially independent of percentage of motile sperm at the start of the incubation for SBA (Fig. 2). All samples with ≤30% motile sperm had <10% sperm bound, but 12 samples with 35 to 70% motile sperm also had <10% sperm bound. For all 76 samples, the correlation between percentage of motile sperm and percentage of sperm bound was 0.526, and for samples with >35% motile sperm, the r value was 0.387. After 60 minutes of incubation at 37°C during SBA, percentage of motile sperm ranged from 10 to 80% (data not shown), but for 53 of the 76 samples it was ≥45%. Although the correlations between sperm motility at the start of SBA and percentage of sperm bound were statistically significant ($P < 0.01$), they were low, and percentage of motile sperm accounted for <30% of the variation in percentage of sperm bound. Clearly (Fig. 2), a spermatozoal attribute(s) other than motility must be involved in sperm binding.

Considering only the first sample from individuals providing multiple ejaculates, there were data for 19 prospective donors at site 1, 16 subjects at site 2, and 12 subjects plus 10 occasional donors at site 3. Data for these 57 samples displayed a wide range in percentage of sperm bound (Fig. 1). There were site-specific differences in percentage of sperm bound (insert, Fig. 1; $P < 0.01$), but the

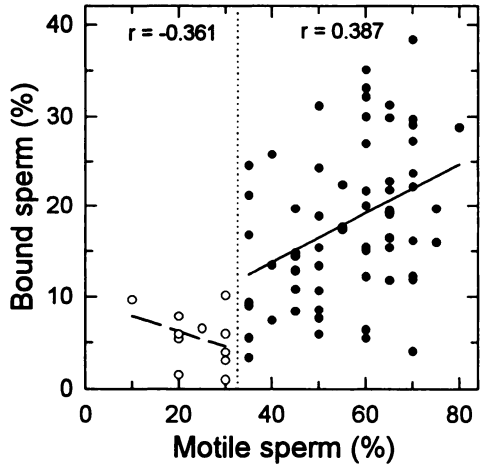

FIG. 2. Relationship between percentage of motile sperm at start of SBA and percentage of sperm bound (n = 76 samples). Considering all data, the correlation was 0.526. No sample with ≤30% motile sperm had >10% sperm bound. For samples with ≤30% motile sperm, there was no positive relationship between the attributes. For samples with >30% motile sperm, the percentage of motile sperm accounted for only 15% of the variation in percentage of sperm bound.

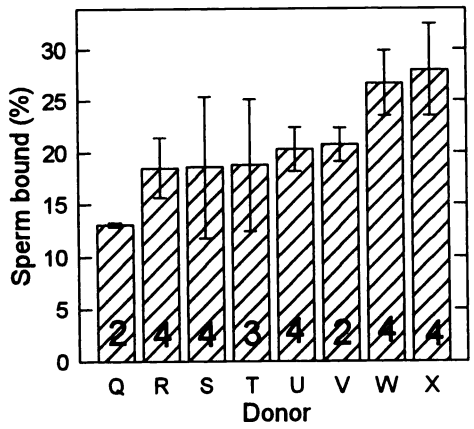

FIG. 3. Range in mean percentage of sperm bound in SBA for fresh semen from occasional seminal donors at site 3. Mean (±SEM) for designated number of ejaculates. Donor means differed ($P < 0.02$).

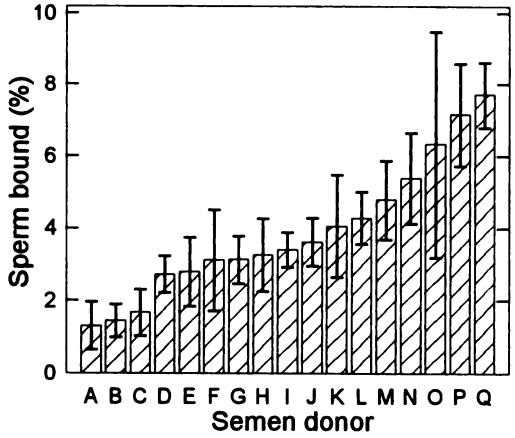

FIG. 4. Range in mean percentage sperm bound in SBA for frozen–thawed semen from commercial seminal donors; semen from site 1. Mean (±SEM) for two or three ejaculates per donor. Donor means differed ($P < 0.03$ for F-ratio in ANOVA).

percentage appeared to be unrelated to status of what we termed occasional donors, prospective donors, or subjects. At site 3, values for subjects and occasional donors averaged 19 and 24% sperm bound, respectively, and for samples with ≥20% sperm bound, seven were from subjects and six were from occasional donors. The difference among sites might reflect the individuals providing semen, the extent of sperm washing before assay (once vs. twice), or random variation, but the real cause is unknown.

An important question is consistency of percentage of sperm bound for several samples from an individual, versus random, sample-to-sample variation. This was evaluated with two to four ejaculates from occasional seminal donors at site 3. It was evident (Fig. 3) that there were true differences ($P < 0.02$) among these individuals, considered *a priori* to provide normal semen. The within-donor (two to four ejaculates each) CV for percentage of sperm bound averaged 31%.

Binding of Percoll-Processed Fresh Sperm

Processing sperm through Percoll or similar medium provides samples with a high percentage of motile sperm. To determine if such processing affected sperm binding, ejaculates from nine subjects (at site 2) were split, and sperm were washed twice (as above) or isolated by centrifugation through Percoll (see Ord et al, 1990), then washed three times. For eight of nine Percoll-processed aliquots, percentage of motile sperm was higher than for the control aliquot when incubation for the SBA started. Means for all nine samples were 80 versus 65% ($P < 0.02$; paired t-test). However, the mean percentage of sperm bound (~9%) was not different for Percoll-processed versus washed samples.

Binding of Thawed Sperm from Cryopreserved Semen

For 17 regular donors previously active at site 1, we evaluated semen in vials representing three different ejaculates from each of 14 donors and representing two ejaculates from each of 3 donors. For the 48 samples, values ranged from <1 to 13% sperm bound; the mean was 3.9%. Considering the 17 donors, there was a >5-fold range in mean percentage of sperm bound (Fig. 4), differences associated with donor were significant ($P < 0.03$), and the donor means also averaged 3.9% sperm bound. The correlation between percentage of motile sperm and percentage of sperm bound was 0.329 ($P < 0.05$; $n = 48$). Hence, with thawed sperm, percentage of motile sperm likely accounted for <11% of the variation in % sperm bound.

Binding for Matched Samples of Fresh Versus Frozen–Thawed Sperm

We compared fresh versus frozen semen for 17 individuals (there was insufficient frozen semen from two other men) being screened as potential semen donors at site 1. As anticipated, percentage of sperm bound was lower for frozen–thawed aliquots of sperm than for fresh sperm from the same ejaculates (Fig. 5); means were 2.2 and 13.3% sperm bound ($P < 0.01$; paired t-test). On the basis of the correlation between percentage of sperm bound for fresh and frozen–thawed aliquots of these 17 ejaculates ($r = 0.527$; $P < 0.05$), we concluded that variation in percentage of sperm bound for fresh semen accounted for <30% of the variation in percentage of sperm bound for frozen–thawed samples ($r^2 = 0.278$).

Discussion

The goal of this study was to evaluate semen from many men for convenience at three sites and not to study semen from male-factor patients. Indeed, semen from all donors,

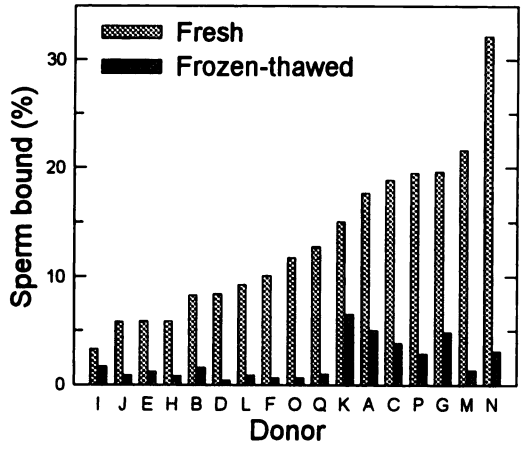

FIG. 5. Comparison of percentage of sperm bound in SBA for fresh versus frozen–thawed sperm in ejaculates from prospective seminal donors at site 1, divided to provide fresh and cryopreserved aliquots. Treatment means differed ($P < 0.01$). As detailed in the text, calculation of binding on the basis of motile sperm placed into wells did not eliminate the difference between fresh and thawed samples.

prospective donors, and 21 of 28 subjects (male members of couples being evaluated for infertility) met WHO (1993) criteria. For the other seven subjects, semen was deficient only in terms of sperm motility (36 to 50% motile sperm and low forward velocity, on the basis of subjective evaluations). No sample was classified as oligospermic or teratozoospermic. Collectively, the 76 samples of fresh semen used in this study likely represented a cross-section of men; they did not represent a population of male-factor men.

The SBA detected, in a repeatable manner (see text; Fig. 3), differences among individuals in quality of sperm in fresh (Fig. 1) and frozen–thawed (Fig. 4) semen and also differences between fresh and frozen–thawed aliquots of the same ejaculates (Fig. 5). Values ranged from <1 to 38% sperm bound for 76 ejaculates of fresh semen and from <1 to 13% sperm bound for 65 freeze codes of cryopreserved semen. Both ranges exceed 10-fold. We concluded that for individuals whose semen contained >30% motile sperm, the SBA detected differences in sperm binding (Fig. 2), and evaluation of this attribute might enhance the basis for decisions on therapy for subfertile couples. To establish predictive value of the SBA, replicate evaluations (≥2 ejaculates per man) of a large number of patients representing a wide range of suspected male-factor subfertility, together with outcome data for fertility or some other gold standard, would be necessary.

To augment correlation analyses (e.g., Fig. 2), the percentage of sperm bound also was calculated on the basis of the number of motile sperm placed into each well, rather than on total sperm, although results likely are confounded. On this basis, the range for the 76 ejaculates of fresh semen was 3 to 96% sperm bound. The rank order was not substantially changed, and the coefficients of determination (r^2 value, percentage of sperm bound attributed to percentage of motile sperm) for fresh or frozen–thawed sperm were 0.276 and 0.108. For this reason and the presentation in Fig. 2, it was obvious that the SBA measures a spermatozoal attribute only moderately influenced by sperm motility.

The exact mechanism involved in binding of human sperm to the substrate of an SBA plate is unknown. The binding substrate is an extract of chicken egg membrane, in which the inner lamina perivitelline has substantial homology with the core region of human and other ZP_3s (Waclawek et al, 1998; Takeuchi et al, 1999), a characteristic hydropathy profile, and eight strictly conserved cysteine residues. Although carbohydrates are involved in fertilization (Yanagimachi, 1994; Benoff, 1997), binding of sperm in this assay need not involve carbohydrates. Binding of human sperm to this substrate is enhanced by brief exposure of sperm to a nonglycosylated synthetic peptide (Amann et al, 1999a,b). Further, there is evidence that the polypeptide backbone of ZP_3 can bind sperm and induce calcium influx and acrosomal changes (Koyama et al, 1991; Brewis et al, 1996; Chapman et al, 1998). Only extensive research can unravel the mechanism(s) by which human or rooster sperm bind to the substrate of an SBA plate or to sheets of the native inner lamina perivitelline.

Is there reason to expect that the SBA, or some similar assay, might be diagnostic for fertility or subfertility? As discussed earlier (Amann and Hammerstedt, 1993; Cummins, 1995; Hammerstedt, 1996), because the path to fertilization is essentially sequential, for each individual spermatozoon, failure at one step precludes success. Hence, correct prediction of subfertility will be easier than correct prediction of fertility. Barbato (1999) generalized that subfertility might be considered as a threshold trait, with individual males falling into one of two phenotypic categories—having or not having the trait. Expression of a threshold trait from an underlying genetic cause (e.g., sperm–egg binding) has an underlying continuous distribution of genetic variation, but phenotypic expression of subfertility occurs only in one tail of the distribution (i.e., the lower <30% of the fertility scale). This is illustrated in figure 4 of Barbato et al (1998). Significantly, heritability of subfertility associated with sperm–egg binding, as measured via the SBA, was relatively high at ~0.38 compared with that of ~0.08 for fertility. Further, the genetic and phenotypic correlations between sperm binding and subfertility (considered <30% of eggs yielding a live chick) were 0.75 and 0.85 (Barbato, 1999). Assuming that similar mechanistic defects exist in other species, it is plausible that use of the SBA with human sperm might be diagnostic for subfertility resulting from a deficiency in sperm–egg binding.

A similar threshold phenotypic response is evident in

data for the hemizona index, a measure of capacity of human sperm to bind to the human zona. The hemizona pellucida binding assay has good discrimination (Coddington et al, 1994). The hemizona index ranges from ~0 to >100, but values greater than ~60 are above the diagnostic range for subfertility because they are near the asymptote of the response curve. A hemizona index ≥60 predicts all oocytes will be fertilized and is almost always correct, an index <40–43 correctly predicts less than 100% fertilized eggs and is correct 83% of the time (true negative prediction, all eggs fertilized, 87% correct), and an index of <14–16 predicts no fertilized oocytes and is correct 82% of the time. For some individuals, failure of sperm to bind in a hemizona assay is associated with general immaturity of sperm, as evidenced by morphology, creatine kinase isoforms, and incomplete remodeling of the plasma membrane (Huszar and Vigue, 1993; Huszar et al, 1994, 1997). Other data (Boué and Sullivan, 1996; Hershlag et al, 1998; Amann et al, 1999a,b; Parent et al, 1999) can be interpreted as supporting the concept that subfertility can result from low expression of one or more threshold traits, such as mannose binding or a deficiency of a specific sperm-bound protein, rather than general cell immaturity.

Obviously, cryopreservation (and/or centrifugation of sperm after thawing rather than as fresh sperm) altered sperm in a manner that reduced their ability to bind to the egg membrane substrate of a SBA plate. This was directly tested using 17 ejaculates that were divided, processed, and evaluated; resultant means were 13 and 2% sperm bound for fresh and frozen–thawed sperm, respectively. The conclusion that cryopreservation reduces ability of sperm to bind is buttressed by data for random samples (not split samples, as in this study, but from different sources) that averaged 17.3% sperm bound for fresh semen (n = 57 ejaculates) versus 3.9% sperm bound for frozen–thawed semen (n = 48 ejaculates). In a companion paper (Amann et al, 1999b), we speculate that reduced ability of thawed sperm to bind to the egg membrane substrate or a hemizona pellucida (see below), is due in part to cryoelution of a sperm–egg binding protein from the plasma membrane.

There are two hemizona comparisons of subsamples of human sperm evaluated before and after freezing and subsequent thawing. Both revealed a decrease in capacity of swim-up samples prepared after thawing to bind in a hemizona assay, as compared with swim-up samples of fresh sperm (Coddington et al, 1991; Morshedi et al, 1995), although there was no difference in percentage of motile sperm or ATP content (Morshedi et al, 1995). With rooster sperm, there also is sharp divergence between the conclusion concerning postthaw quality when based on percentage of motile sperm versus when based on binding of sperm to an egg membrane substrate or on actual fertility of sperm (Barbato et al, 1998). Sperm binding was diagnostic, but percentage of motile sperm was not. Thus, it is evident that reliance on visual estimates of sperm motility or even on CASA evaluations of sperm motion is insufficient to detect all damage associated with cryopreservation or to accurately predict which samples of human sperm are likely to provide a relatively high fecundity rate.

The hemizona index perhaps is the best test currently available to predict failure of sperm in IVF. In a recent compilation (Oehninger et al, 1997), it was reported that a hemizona index cutoff of ≤30 enabled correct prediction of an oocyte fertilization rate of <60% in IVF for 87% of cases, with false positive and negative rates of 27 and 6%. The overall rate of misclassification was 14%, based on 361 IVF cycles. The SBA reported herein measures sperm binding, as does the hemizona assay. Nevertheless, a positive result in an SBA (i.e., low percentage of sperm bound) might not be predictive for relatively low fertility with a high predictive value, sensitivity, and specificity. However, on the basis of data presented, it would be appropriate to evaluate diagnostic potential by directly comparing percentage of sperm bound in the SBA with the hemizona penetration index and IVF outcome, using a series of ejaculates representing both normal and subfertile men. This could establish diagnostic value of the SBA for predicting failure of sperm–zona binding, which should be an important criterion in advising couples in choosing between intrauterine insemination or IVF versus intracytoplasmic sperm injection to provide an embryo(s) for transfer into the uterus.

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References

Amann RP, Hammerstedt RH. In vitro evaluation of sperm quality: an opinion. *J Androl* 1993;14:397–406.

Amann RP, Hammerstedt RH, Shabanowitz RB. Exposure of human, boar, or bull sperm to a synthetic peptide increases binding to an egg-membrane substrate. *J Androl* 1999a;20:34–41.

Amann RP, Shabanowitz RB, Huszar G, Broder SJ. Increased in vitro binding of fresh and frozen-thawed human sperm exposed to a synthetic peptide. *J Androl* 1999b;20:655–660.

Barbato GF. Genetic relationships between selection for growth and reproductive effectiveness. *Poult Sci* 1999;78:444–452.

Barbato GF, Cramer PG, Hammerstedt RH. A practical in vitro sperm-egg binding assay that detects subfertile males. *Biol Reprod* 1998;58:686–699.

Benoff S. Carbohydrates and fertilization: an overview. *Mol Hum Reprod* 1997;3:599–637.

Boué F, Sullivan R. Cases of human infertility are associated with the absence of P34H, an epididymal sperm antigen. *Biol Reprod* 1996; 54:1018–1024.

Brewis IA, Clayton R, Barratt CL, Hornsby DP, Moore HD. Recombinant human zona pellucida glycoprotein 3 induces calcium influx and acrosome reaction in human spermatozoa. *Mol Hum Reprod* 1996;2: 583–589.

Chapman N, Kessopoulou E, Andrews P, Hornby D, Barratt C. The polypeptide backbone of recombinant human zona pellucida glycoprotein-3 initiates acrosomal exocytosis in human spermatozoa in vitro. *Biochem J* 1998;330:839–845.

Coddington CC, Franken DR, Burkman LJ, Oosthuizen WT, Kruger T, Hodgen GD. Functional aspects of human sperm binding to the zona pellucida using the hemizona assay. *J Androl* 1991;12:1–8.

Coddington CC, Oehninger SC, Olive DL, Franken DR, Kruger TF, Hodgen GD. Hemizona index (HZI) demonstrates excellent predictability when evaluating sperm fertilizing capacity in in vitro fertilization patients. *J Androl* 1994;15:250–254.

Cummins JM. Tests of sperm function. In: Grudzinskas JG, Yovich JL, eds. *Gametes—The Spermatozoon*. Cambridge, United Kingdom: Cambridge University Press; 1995:70–103.

Franken DR, Acosta AA, Kruger TF, Lombard CJ, Oehninger S, Hodgen GD. The hemizona assay: its role in identifying male factor infertility in assisted reproduction. *Fertil Steril* 1993;59:1075–1080.

Hammerstedt RH. Evaluation of sperm quality; identification of the subfertile male and courses of action. *Anim Reprod Sci* 1996;42:77–87.

Hershlag A, Scholl GM, Jacob A, Mandel FS, Guhring P, Paine T, Cooper GW, Benhoff S. Mannose ligand receptor assay as a test to predict fertilization in vitro: a prospective study. *Fertil Steril* 1998;70:482–491.

Huszar G, Sbracia M, Vigue L, Miller DJ, Shur BD. Sperm plasma membrane remodeling during spermiogenetic maturation in men: relationship among plasma membrane β1,4-galactosyltransferase, cytoplasmic creatine phosphokinase, and creatine phosphokinase isoform ratios. *Biol Reprod* 1997;56:1020–1024.

Huszar G, Vigue L. Incomplete development of human spermatozoa is associated with increased creatine phosphokinase concentration and abnormnal head morphology. *Mol Reprod Dev* 1993;34:292–298.

Huszar G, Vigue L, Oehninger S. Creatine kinase immunocytochemistry of human sperm-hemizona complexes: selective binding of sperm with mature creatine kinase-staining pattern. *Fertil Steril* 1994;61: 136–142.

Knapp RG, Miller MC 3rd. *Clinical Epidemiology and Biostatistics*. Philadelphia, Pennsylvania: Williams and Wilkins; 1992.

Koyama K, Hasegawa A, Inoue M, Isojima S. Blocking of human sperm-zona interaction by monoclonal antibodies to a glycoprotein family (ZP4) of porcine zona pellucida. *Biol Reprod* 1991;45:727–735.

Morshedi M, Oehninger S, Blackmore P, Bocca S, Coddington C, Hodgen G. Investigation of some biochemical and functional effects of cryopreservation of human spermatozoa using an automated freezing-quick-thawing method. *Int J Androl* 1995;18:279–286.

Oehninger S. Diagnostic significance of sperm/zona pellucida interaction. *Med Reprod Rev* 1992;1:57–87.

Oehninger S, Mahony M, Özgür K, Kolm P, Kruger T, Franken D. Clinical significance of human sperm-zona pellucida binding. *Fertil Steril* 1997;67:1121–1127.

Ord T, Patrozio P, Marello E, Balmaceda JP, Asch RH. Mini-Percoll: a new method of semen preparation for IVF in severe male factor infertility. *Hum Reprod* 1990;5:987–989.

Parent S, Lefièvre L, Brindle Y, Sullivan R. Bull subfertility is associated with low levels of a sperm membrane antigen. *Mol Reprod Dev* 1999; 52:57–65.

Takeuchi Y, Nishimura K, Aoki N, Adachi T, Sato C, Kitajima K, Matsuda T. A 42-hDa glycoprotein from chicken egg-envelope, an avian homolog of the ZPC family of glycoproteins in mammalian zona pellucida. *Eur J Biochem* 1999;260:736–742.

Töpfer-Petersen E, Calvete JJ. Molecular mechanisms of the interaction between sperm and the zona pellucida in mammals: studies on the pig. *Int J Androl (Suppl 2)* 1995;18:20–26.

Töpfer-Petersen E, Calvete JJ, Sanz L, Sinowatz F. Carbohydrate- and heparin-binding proteins in mammalian fertilization. *Andrologia* 1995;27:303–324.

Waclawek M, Foisner R, Nimpf J, Schneider WJ. The chicken homologue of zona pellucida protein-3 is synthesized by granulosa cells. *Biol Reprod* 1998;59:1230–1239.

Windt ML, Franken DR, Kruger TF, Oehninger SC. In vitro fertilization failure: identification of gamete defects by investigation of sperm-zona pellucida binding capacity of unfertilized oocytes. *Andrologia* 1996;28:211–215.

World Health Organization. *WHO Laboratory Manual for the Examination of Human Semen and for Sperm-Cervical Mucous Interaction*. 3rd ed. Cambridge, United Kingdom: Cambridge University Press; 1993.

Yanagimachi R. Mammalian fertilization. In: Knobil E, Neill JD, eds. *The Physiology of Reproduction*. New York: Raven Press; 1994:189–317.