

Relationship between the Human Sperm Hypo-osmotic Swelling Test and Sperm Penetration Assay

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ABSTRACT: The hypo-osmotic swelling (HOS) test has been proposed as a useful assay in the diagnosis of the infertile male. A good correlation between the HOS test and the sperm penetration assay (SPA) in fertile and normal semen samples was initially found, but subsequently, no significant correlation was demonstrated with fertile and infertile patients. To validate the potential clinical usefulness of the HOS test, we evaluated 92 ejaculates using the HOS test, SPA, and traditional semen parameters. The methodology originally described by Jeyendran et al (1984) was used for the HOS test. The SPA was performed by the original procedure using an 18-hour preincubation period, and for 28 ejaculates, a modified procedure using TEST-yolk buffer was performed. Values of 60% or more for the HOS and 1% or more for the SPA were considered positive, and less than 60% for HOS and 0% for SPA were considered negative when the standard SPA was performed. For the TEST-yolk buffered SPA, values of 20% or more were considered positive. The sensitivity of the HOS test was 87%, but the specificity was 36%. The association of the two tests over and above that expected by chance ($Kappa$) was only 0.23. Using logistic regression, both

sperm count ($P < 0.001$) and morphology ($P < 0.025$) were significant predictors of the SPA classification, but the HOS test did not improve the predictive results ($P > 0.50$). Analysis of distribution of HOS test scores for "fertile" (SPA > 0%) and "infertile" (SPA = 0) samples showed such a wide overlap that it was impossible to designate a threshold value for a HOS test score that is predictive of a positive SPA result. The HOS test by itself has a modest ability to predict SPA. This was indicated by the modest correlation ($r = 0.32$) that, although highly significant ($P = 0.0017$), accounts for only about 10% of the total variation in SPA. The correlation between HOS and SPA results was related to SPA methodology since TEST-yolk buffer-enhanced SPA results provided a slightly higher correlation coefficient ($r = 0.42$). The HOS test does not appear to be a significant predictor of SPA results after considering other measurements.

Key words: Fertility evaluation, hypo-osmotic swelling (HOS) test, sperm penetration assay (SPA).

J Androl 1991;12:152-158.

It is generally accepted that a complete assessment of the human sperm's ability to fertilize human eggs is not achieved with any single biologic test among the many that are currently available. Each test measures only a few sperm functions and provides limited information about the sperm's ability to fertilize an egg. The hypo-osmotic swelling (HOS) test was introduced in 1984 (Jeyendran et al) as a potentially useful assay for evaluating the functional integrity of human spermatozoal membranes. They proposed incorporation of the HOS test into the standard semen analysis for diagnosing male infertility. The test exposes semen samples to hypo-osmotic stress and determines their responses under these conditions, which are detectable by the curling of the tail fibers within the tail plasma membrane.

In theory, a clinical test should give information about the reproductive potential of the sperm. To determine this ability, comparisons with established assays need to be per-

formed. A major development in the evaluation of sperm functions was the discovery that zona-free hamster eggs could be penetrated by capacitated and acrosome-reacted sperm (Yanagimachi et al, 1976, Rogers et al, 1979). Different investigators have compared HOS test results with basic semen parameters and sperm penetration into zona-free hamster eggs (Jeyendran et al, 1984; Chan et al, 1985; van Kooij et al, 1986; Wang et al, 1988; Coetzee et al, 1989; Chan et al, 1989). Based on a small group of selected fertile or normal semen samples, Jeyendran and co-workers reported a good correlation between the HOS test and sperm penetration assay (SPA) results (Jeyendran et al, 1984). Chan and associates evaluated a heterogeneous population of fertile and infertile men and found no significant correlation between these two tests (Chan et al, 1985). Subsequent investigations have been both positive (Coetzee et al, 1989) and negative (van Kooij et al, 1986). To further assess the potential usefulness of the HOS test in a clinical setting, we evaluated a large male population of infertility patients and donors, comparing the relationship between the results of the HOS test with the SPA assay and with standard semen parameters. Our preliminary results were reported previously (Bastias et al, 1987).

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Received for publication July 30, 1990; accepted for publication November 12, 1990.

Materials and Methods

One hundred and eight human semen samples were collected from 92 men. The group included 5 fertile donors and 87 partners of infertile couples with unknown causes of infertility at the time of this study. Samples were collected by masturbation and allowed to liquefy for 30 minutes at room temperature. A routine semen analysis was performed to determine sperm count, motility, and morphology. Values were considered normal for a count of 20×10^6 sperm/ml or more, a motility of 50% or more, and a standard morphology of 50% or more, which were evaluated according to World Health Organization protocol.

An aliquot of semen was also taken to perform the HOS test as originally described (Jeyendran et al, 1984). Undiluted ejaculate (0.1 ml) was mixed with 1 ml of hypo-osmotic solution (150 mOsm/kg) prepared with 75 mmol/L fructose and 25 mmol/L sodium citrate. The sperm suspension was incubated at 37°C for at least 30 minutes and no longer than 3 hours. A drop of the sample was placed on a glass slide and observed using a phase-contrast microscope (400 \times) for curling of the sperm tail. A minimum of 100 sperm was observed, and the percentage of swollen sperm was calculated as $100 \times$ the number of swollen tails divided by the total number of sperm examined. Since it is likely that one might find spermatozoa with curled tails before exposure to the hypo-osmotic medium, the samples were examined before treatment and the percentage of sperm with curled tails was subtracted from the percentage obtained after treatment. Values for the HOS test of 60% or more were accepted as normal and less than 60% were considered abnormal. Alternately, data were also analyzed using 50% as the lower limit for positive results.

To evaluate the relationship of the HOS test to the SPA (Rogers et al, 1979), the semen sample was transferred to a sterile centrifuge tube and diluted to 10 ml with Biggers, Whitten, and Whittingham solution (BWW). Spermatozoa were pelleted by centrifugation at 600g for 10 minutes and subsequently washed twice with 10 ml BWW. The final sperm pellet was resuspended in 1 ml of BWW, counted to adjust the sperm concentration to 10×10^6 sperm/ml, and capacitated for 18 to 20 hours at 37°C in an air incubator. Hamster oocytes were treated with hyaluronidase and trypsin to remove the cumulus and zona pellucida, respectively (Rogers et al, 1979), and were then transferred into 100 μ l droplets of the sperm suspension under paraffin oil. After 2 hours incubation in an air incubator, at least 30 oocytes were examined by phase-contrast microscopy for the presence of decondensing sperm heads with attached or closely associated tails. Values of 1% or greater penetration were considered positive and 0% penetration as negative. A subset of patients was tested using the TEST-yolk buffer (TES [N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid], Tris, chicken egg yolk, glucose, and antibiotics) modification of the SPA (Falk et al, 1990). With this methodology, a positive score is defined as 20% or greater penetration. To provide an opportunity for the HOS test to perform as well as possible, other criteria for normal and abnormal results were used for the SPA tests as well.

Several statistical techniques were used to assess the relationship between the HOS test and SPA. Spearman rank correlation was used to assess whether there was any association between the level of any measurements and SPA. The Wilcoxon rank sum test was used for comparisons between groups. Sensitivity and speci-

ficity of the HOS test as a diagnostic test for results with SPA were assessed. Sensitivity is the percentage of abnormal SPA results found to be abnormal with the HOS test, and specificity is the percentage of normal SPA results found to be normal with the HOS test. Several different criteria were used for each test to allow us to assess the best performance of the HOS test as an indicator of SPA test results. The overall association between the two classifications (normal or abnormal for the HOS test vs. normal or abnormal for SPA) were assessed using the Kappa statistic (Fleiss, 1981). Kappa measures the association between the two outcomes over and above the association expected by chance, based on the proportions of normal and abnormal for each test separately. Values of Kappa above 0.75 indicate a good association, and values below 0.40 indicate a poor association.

We also used logistic regression to assess the importance of the HOS test as a predictor of SPA after allowing for other measurements in a routine sperm analysis. The measurements available were sperm count, percentage with normal motility, and percentage with normal morphology. A stepwise approach was used to identify the most significant factors.

Results

Data were available from 108 measurements on 92 individuals. Thirty measurements using the TEST-yolk SPA system were available on 28 individuals. Because the statistical tests used assume that all observations are independent, only one observation was used for each individual. When there was more than one record for an individual, observations with measurements for the TEST-yolk SPA system were preferentially selected; otherwise, the first observation for an individual was used. The exclusion of additional observations, necessary for a valid analysis, had no substantive impact on the results below.

Results of the SPA assay ranged from 0% to 97%, with a mean of 15% ($\pm 22\%$, SD). Sperm count ranged from 1×10^6 to 218×10^6 , with a mean of $69 \pm 53 \times 10^6$. Motility averaged $45 \pm 6\%$ (range, 4% to 84%), normal morphology averaged $56 \pm 9\%$ (range, 33% to 76%), and the HOS test averaged $46 \pm 20\%$ (range, 5% to 85%). For the TEST-yolk system, SPA ranged from 0% to 100%, with average of $44 \pm 35\%$.

Association of Measurements from Sperm Analysis

Table 1 shows the Spearman rank correlation between each of the five measurements available on all 92 observations. The correlation of SPA with the HOS test, morphology, and motility were all similar and substantially less than the association of SPA with total sperm count. The HOS test was highly correlated with motility and sperm count, and less correlated with SPA results and morphology. All correlations between variables were statistically significant, however ($P < 0.05$). Figure 1 shows the scatter plots of the HOS test results with the other measurements available on all 92 observations.

The correlation between the TEST-yolk system and the

Table 1. Spearman correlation between measurements in sperm analysis

	SPA	Concentration	Motility	Morphology
Concentration	0.50 0.0001			
Motility	0.31 0.0026	0.34 0.0009		
Morphology	0.31 0.0029	0.21 0.0455	0.36 0.0004	
HOS	0.32 0.0016	0.48 0.0001	0.51 0.0001	0.26 0.0129

Entries are the correlation coefficient (r) and the P value.

The SPA values were obtained using the original methodology with BWB preincubation.

regular SPA was $r = 0.44$ ($P = 0.0199$; $n = 28$). The next best correlation with the results of the TEST-yolk SPA system was $r = 0.42$ ($P = 0.0276$) with the HOS test. These results were based on 28 observations. The correlations for the other sperm variables with the TEST-yolk SPA were less than 0.31 and were not statistically significant.

Diagnostic Accuracy of the HOS Test as a Predictor of SPA Outcome

Table 2 shows the sensitivity and specificity of the HOS test as a predictor of SPA using several criteria for normal and abnormal results for each test. Also shown is the number of observations excluded, if necessary, because of the use of an indeterminate result for the HOS test as suggested by Jeyendran et al (1984). Results for Kappa, showing the association over and above that which would be expected by chance, are also shown in the table.

As Table 2 shows, the sensitivity ranged from 78% to 87% for the regular SPA test but was 100% for the TEST-yolk SPA test. Specificity, however, exceeded 50% only when HOS test results between 50% and 59% were classified as indeterminate. This led to almost 20% of the total observations being excluded from consideration. Kappa exceeded 0.40, a minimal value indicating fair association, only in one of the ten combinations considered. This occurred when HOS test results from 50% to 59% were excluded; 8 of the 28 observations (29%) available for this analysis were excluded. SPA was assessed as normal for results 20% or greater and abnormal otherwise using the TEST-yolk system. For this combination, sensitivity was 100% and specificity was 67%. Kappa was only 50% in this combination.

Prediction of Normal/Abnormal Results of SPA Using Multiple Variables

A multiple logistic regression model was used to predict the results of SPA based on other variables measured. For the standard SPA, the sperm count was clearly the most important predictor for each of the three criteria of abnormality

used ($P < 0.001$). When abnormal was defined as 0% only, then the percentage with normal morphology was also strongly associated with SPA outcome after adjustment for the sperm count ($P < 0.025$). The results for the HOS test were not statistically significant predictors after adjustment for sperm count, regardless of whether morphology was included in the model ($P > 0.15$ for all analyses). When SPA was assessed using the TEST-yolk system, the HOS test was the most important predictor of outcome ($P = 0.0349$). No other variables were significant predictors of SPA with this system.

Relationship Between the HOS Test and SPA for Penetrators and Nonpenetrators

Of 92 samples evaluated in our study, the average HOS test value for 45 men that gave negative SPA results (0% penetration) was $41.04 \pm 19.65\%$, which was significantly lower ($P = 0.0124$) than $51.40 \pm 18.22\%$ for 47 men who had positive SPA results (>0% penetration). The distributions of the percentage of swollen sperm (HOS test score) from "fertile" and "infertile" ejaculates, as defined by the SPA result, are shown in Figure 2. A wide range of scores was observed in both fertility categories. The considerable overlap does not allow the designation of a threshold value for HOS test score that is predictive of the SPA result.

Discussion

Since semen analysis reveals severe alterations in spermatogenesis and disorders in sperm maturation, it is difficult to assess fertility based on the standard semen parameters of count, motility, and morphology. To obtain more information on the functional reproductive quality of spermatozoa, the HOS test was proposed as an easy procedure to detect physiologic integrity of the sperm membrane (Jeyendran et al, 1984). This assay assesses the sperm membrane's functional integrity by evaluating its reaction under hypo-osmotic conditions. We compared results from the HOS test with the zona-free hamster egg penetration assay since sperm-egg fusions can occur only in the presence of structurally and functionally normal membranes of capacitated and acrosome-reacted sperm (Yanagimachi et al, 1976). The HOS test may be an indicator of the fertilizing ability of spermatozoa and could correlate with the fertilizing capacity as assessed with the SPA. Such a comparison was initially reported by Jeyendran (1984) who found a good correlation ($r = 0.94$) between SPA and the HOS test in a small group of men ($n = 23$) with normal semen samples. Chan et al (1985) could not reproduce these results when a heterogeneous population of 171 patients who attended an infertility clinic were tested. An insignificant correlation between sperm swelling and *in vitro* sperm fertilizing ca-

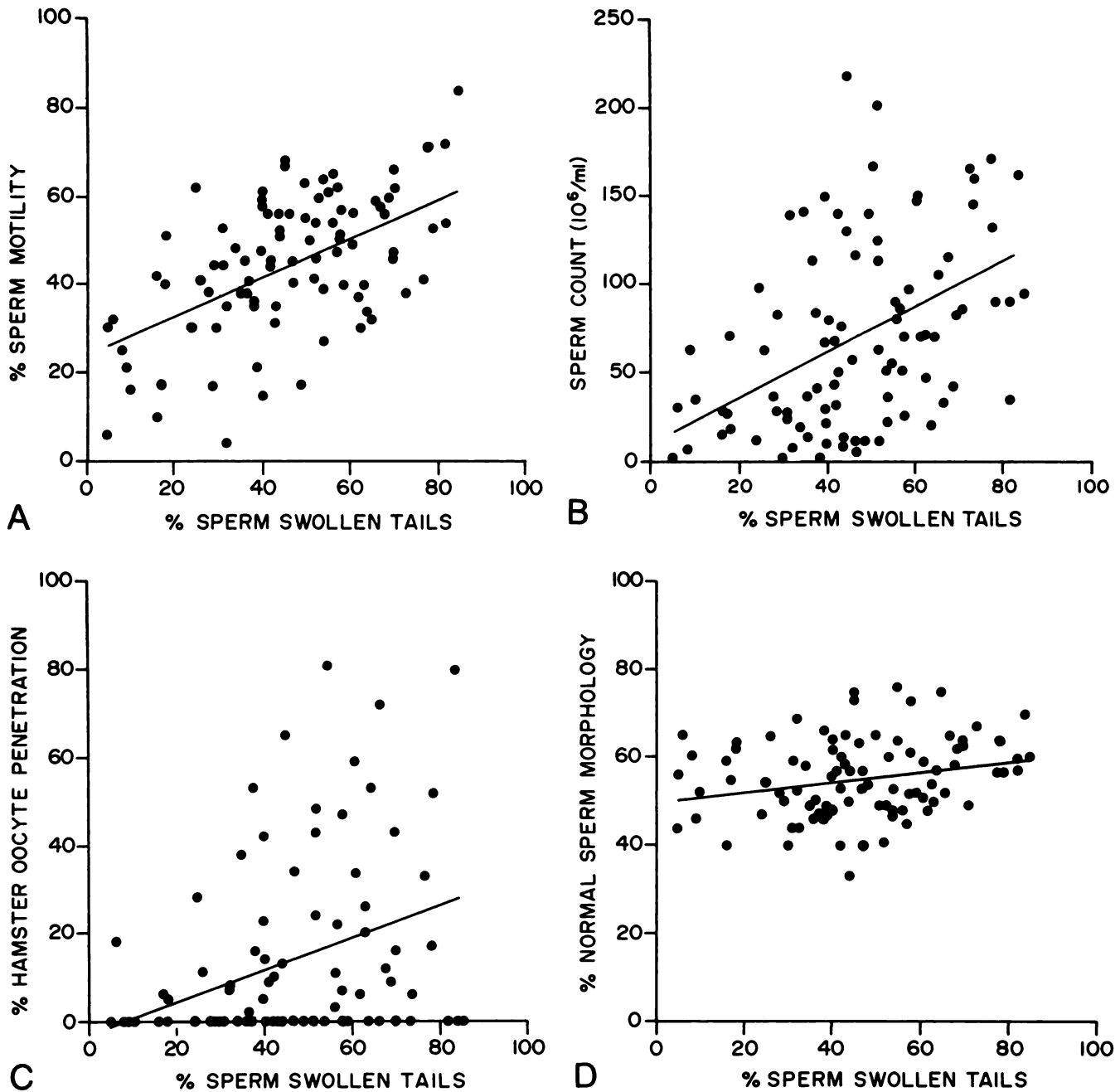


FIG. 1. Relationship between the percentage of sperm swollen tails and other sperm characteristics. (A) Percentage of sperm motility; (B) sperm count; (C) percentage of sperm penetration into zona-free hamster eggs; and (D) percentage of sperm with normal morphology. SPA values were obtained using the original methodology with BWW preincubation.

capacity, as assessed by the zona-free hamster ovum penetration assay, was found.

Since the original challenge by Chan and co-workers (1985), other investigators have looked at the relationship between the HOS test and SPA. In 1986, van Kooij et al found no significant correlation between the hamster ova test and swelling of the spermatozoa in a hypo-osmotic medium (van Kooij et al, 1986). In their study, 73 men

consulting for infertility were evaluated, and they concluded that the "swell test" was a quick and simple test to evaluate membrane integrity without having strong prognostic value for fertilization. Two years later, Wang et al (1988) compared results of semen analyses, SPA, the HOS test, and ATP levels in groups of fertile and infertile men. They found that the SPA and HOS test were capable of providing significant information on the fertility status of

Table 2. Diagnostic accuracy of HOS as a predictor of SPA results

Diagnostic criteria: HOS (%)	Diagnostic criteria: SPA (%)	Sensitivity (%)	Specificity (%)	Number excluded	Kappa
Regular SPA (n = 92)					
Normal: ≥ 60 Abnormal: < 60	Normal: > 0	87	36	0	0.23
	Abnormal: 0				
Normal: ≥ 60 Abnormal: < 60	Normal: ≥ 10	84	40	0	0.26
	Abnormal: < 10				
Normal: ≥ 60 Abnormal: < 60	Normal: ≥ 20	82	44	0	0.27
	Abnormal: < 20				
Normal: ≥ 60 Abnormal: < 60	Normal: > 0	83	45	18 (20%)	0.28
	Abnormal: 0				
Normal: ≥ 60 Abnormal: < 60	Normal: ≥ 10	80	50	18 (20%)	0.32
	Abnormal: < 10				
Normal: ≥ 60 Abnormal: < 60	Normal: ≥ 20	78	58	18 (20%)	0.34
	Abnormal: < 20				
TEST-yolk SPA (n = 28)					
Normal: ≥ 60 Abnormal: < 60	Normal: > 0	100	40	0	0.13
	Abnormal: 0				
Normal: ≥ 60 Abnormal: < 60	Normal: ≥ 20	100	50	0	0.36
	Abnormal: < 20				
Normal: ≥ 60 Abnormal: < 60	Normal: > 0	100	56	8 (29%)	0.20
	Abnormal: 0				
Normal: ≥ 60 Abnormal: < 60	Normal: ≥ 20	100	67	8 (29%)	0.50
	Abnormal: < 20				

the patients. They did not believe, however, that the addition of the results from these tests to routine semen analysis significantly improved the predictability of fertility. More recently, Coetzee (1989) reported weak correlations between the HOS test and SPA ($r = 0.42$, $P < 0.0016$), and the HOS test and human *in vitro* fertilization (IVF; $r = 0.24$, $P = 0.0560$). Thirteen of the 16 patients (81.2%) whose sperm were unable to penetrate a single zona-free hamster egg had normal HOS ($> 60\%$). They concluded that the HOS test has a limited predictive value. Despite this conclusion, they suggested that a HOS test result of less than 50% was a definite indicator of a male factor. Unfortunately, in their SPA ($n = 55$) and IVF ($n = 63$) groups, only two samples penetrated less than 50% in the HOS test. Greater numbers of patients with low HOS test scores are required to make any meaningful conclusions.

Our results show a low but statistically significant correlation between SPA and the HOS test ($r = 0.32$, $P = 0.0016$). We therefore agree with Jeyendran et al and Coetzee et al. An explanation for the lack of a direct correlation between the HOS test and SPA was given recently by deCastro et al (1990). They suggested that a direct correlation between the HOS test and SPA is often absent or weak when ejaculates from infertility patients are tested because these ejaculates frequently suffer from more than one ab-

normality. Another explanation for the lack of correlation may be the SPA methodology selected by the investigators. The potential for variability in results due to differences in methodology is obvious (Table 3). No two investigators who compared the HOS test with SPA used the same combination of albumin type, albumin concentration, sperm preincubation time, or sperm and egg incubation time. These assay variables can have a considerable impact on the results. In confirming or refuting someone's work, care should be taken to perform the tests using identical methodology.

Despite using a longer sperm incubation time than Chan, we found a low significant correlation between SPA and the HOS test ($r = 0.32$, $P < 0.0016$) when evaluating large groups of semen samples from both fertile and infertile men using the standard SPA methodology. In our study of 92 men, a standard SPA result was considered negative when no sperm penetration was observed. This definition of subfertility in the SPA is consistent with Aitken et al (1984) who suggested that only 0% penetration in the SPA is of diagnostic value for infertility, since no pregnancy was reported in the follow-up for that group. In the subset of 28 patients who were tested with two types of SPA methodology (standard and TEST-yolk), the correlation between the HOS test and SPA was enhanced by the use of the TEST-

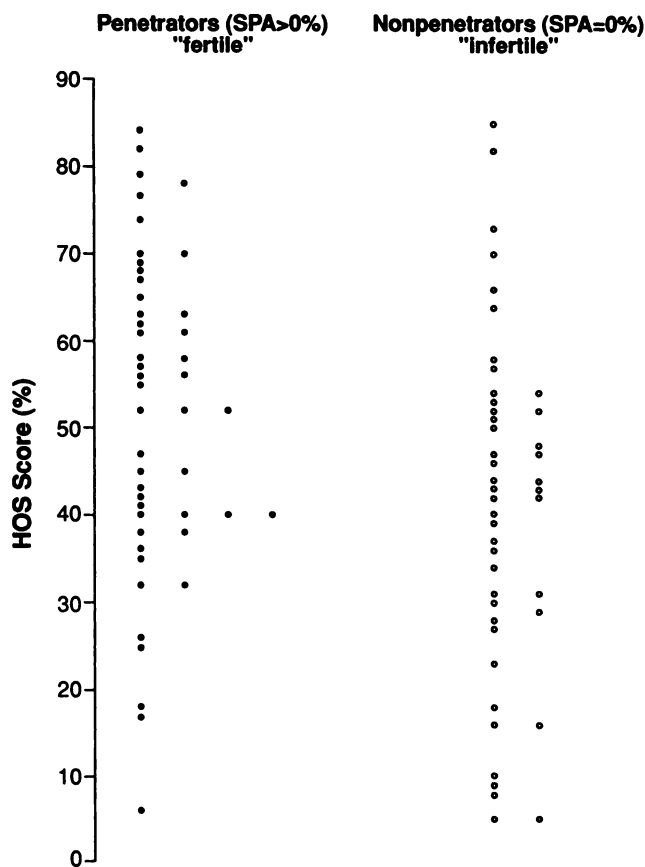


FIG. 2. Distribution of the percentage of swollen sperm (HOS score) from "fertile" and "infertile" ejaculates. Penetrators (n = 47) are defined as those that had a positive penetration rate (>0%). Nonpenetrators (n = 45) are defined as those that had a negative penetration rate (0%).

yolk in the SPA. This result prompts the speculation that SPA methodology may not have been optimized in the studies that found no correlation between the SPA and the HOS test.

Evidence evaluating the usefulness of the HOS test as an indicator of sperm function also comes from IVF studies. In 1986, van der Ven et al originally reported that HOS test values greater than 60% correlated with human oocyte fer-

tilization and values less than 60% were associated with failure in IVF. Since then, other investigators have looked at that question. Sjoblom and Coccia (1989) concluded that the HOS test has limited capability in identifying nonfertilizing samples. This conclusion was based on the HOS test and IVF results of a group of 50 couples. The HOS test was not more useful than any of the standard semen variables in identifying nonfertilizing sperm samples. In fact, the correlation coefficient for percent swell in HOS versus percent fertilization in IVF was not strong (0.399, P not stated). Of the five cases that failed in IVF, only one had an HOS test score of less than 50. Of the seven cases that had an HOS test score less than 50%, five fertilized in IVF. These data suggest that there is not a useful limit or threshold for the percentage of swollen sperm that differentiates between fertilizing and nonfertilizing sperm samples in IVF. Using logistic regression analysis, Liu et al (1988) found no relationship between the HOS test score in semen and fertilization in IVF for 106 couples. In 1989, Barratt reported the results of HOS tests in 56 IVF couples (Barratt et al, 1989). They concluded that "the HOS test was of no predictive value." They found no difference between the HOS test score of fertile and infertile ejaculates (69 vs. 70). They pointed out that the HOS test as a sperm function test is probably limited, and the correlation to physiologic processes necessary for fertilization, such as capacitation, lateral head displacement, acrosome reaction, and the capacity to generate a fusogenic equatorial segment, is as yet undermined.

Limited *in vivo* data are available to support the usefulness of the HOS test. However, Check (1988) reported on HOS tests for a group of 40 men to demonstrate a correlation with pregnancy. Of this group, 33 initiated pregnancies. The HOS test results in the pregnancy group were 28 normal, 5 grey zone (50% to 59% swelling), and none subnormal. Only four patients had subnormal (≤ 50) HOS tests and none of these initiated pregnancies. The authors concluded that larger numbers of patients with normal semen specimens and subnormal HOS tests are needed before any definite conclusions on the benefit of the HOS test in this situation can be made. In 1989, Check et al reported further that the HOS test was highly predictive of eventual achievement of pregnancy in a group of 135 couples. They found that no women conceived whose partner's HOS test score was less than 50%. Interestingly, however, only 13 of the 135 men in their group had an abnormal HOS test score. Furthermore, in the group of 28 men who failed to initiate a pregnancy, 15 (53.6%) had normal or grey zone HOS tests. It appears from these data that a negative HOS is more predictive than a positive HOS test score.

In the specification of normal and abnormal ranges for the HOS test, we initially did not exclude cases falling within the "grey area" (between 50% and 59% of swollen tails; Table 1) as reported by Jeyendran et al in 1984. When the data were reanalyzed after deleting these cases, about

Table 3. Variable SPA methodology in HOS studies

Investigator and year	Albumin (mg/ml)	Sperm preincubation time (n)	Sperm and egg coincubation (n)
Jeyendran et al, 1984	35 HSA	2-3	5
Chan et al, 1985 (Method 1983)	3 BSA	5	6
van Kooij et al, 1986	30 BSA	3	4
Coetzee et al, 1989	3 HSA	18-20	3
Rogers et al, 1990	3 BSA	18-20	2

one fifth of the patient population had to be excluded (Table 2) and the specificity was still under 60%.

The validity of the HOS test for diagnostic purposes has been questioned because correlations between the percentage of swollen spermatozoa in the HOS test and the percentage of penetration in the zona-free penetration test (SPA) were not found for ejaculates of infertility patients and because the differences in mean HOS test values between presumably fertile and infertile men were not large. Part of the explanation for this may be that the classification of individuals as "fertile" or "infertile" is erroneous. A proportion of male patients that came to the clinic for infertility were indeed fertile since a female factor was the primary problem. Furthermore, some men may be categorized as fertile because they have fathered a child even though they have abnormal semen characteristics. In addition, lack of fertility could be due to a variety of sperm factors, only one of which is membrane integrity.

A number of defects may render spermatozoa incapable of penetrating zona-free hamster oocytes. Only some of these defects are measured by the standard semen variables and the HOS test. Individual spermatozoa may suffer from more than one defect, and different sperm populations in the same ejaculate may have different defects. Therefore, it is not surprising that measurement of a single defect, whether it is motility, morphology, sperm number, or membrane integrity, cannot always predict the outcome of a SPA since the absence of a particular defect does not exclude the presence of another. However, if an assay has validity as an indicator of penetration (in SPA), then penetration should be decreased or absent when the assay gives negative results. As the current data indicate, this is the case for the HOS test.

Acknowledgments

The authors thank Alan Buck, Tina DeBoer, Melinda Hinson, and Tom Thompson for technical assistance and Beverly Steele for manuscript preparation.

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11th International Symposium of Operative Andrology

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