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Review

Hypo-Osmotic Swelling Test and Male Factor

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Abstract: For over 30 years, defects of the functional integrity of the sperm membrane, as evidenced by a low hypo-osmotic swelling test when evaluating the semen analysis, are not only associated with male infertility (even with sperm that otherwise seem normal), but unless corrected, successful intrauterine pregnancies will rarely ensue. This defect, interestingly, does not impair fertilization of the oocyte, but instead, prevents a normal-appearing embryo from successfully implanting. The frequency in infertile couples increases with advancing age of the male, ranging from 5% in younger males to 25% in men in their late forties or early fifties. It seems to be related to a toxic protein added to the sperm as they traverse the ejaculatory ducts. The defect is very correctable, either by treating the sperm with the protein digestive enzyme chymotrypsin prior to intrauterine insemination and avoidance of unprotected sex prior to ovulation, or in vitro fertilization with intracytoplasmic sperm injection. Unfortunately, this very inexpensive, easy-to-perform test is rarely performed by the large majority of physicians treating infertility. The purpose of this manuscript is to hopefully rekindle interest within the infertility community to add this test to the standard semen analysis.

Keywords: embryo implantation defect; functional integrity of the sperm membrane; toxic sperm protein; zona pellucida; intracytoplasmic sperm injection; abnormal sperm parameters

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1. Introduction

The Development of the Hypo-Osmotic Swelling (HOS) Test

The functional integrity of the sperm membrane is believed to be an important factor in the acrosome reaction, sperm capacitation, sperm metabolism, and the binding of the sperm to the zona pellucida. Thus, the HOS test was developed in order to evaluate the functional integrity of the sperm membrane, and was first developed to evaluate mammalian sperm, especially those of bulls [1–4]. The principle is as follows: if there are two different concentrations of water on either side of a membrane, the water will seek equilibrium through osmosis. The water from the higher concentration on one side of the membrane will be pumped across the membrane to the one with the lower concentration of water by active transport, until equilibrium is established. Thus, if sperm is placed in a hypo-osmotic solution, if the sperm membrane is functionally intact, the water outside the sperm with a higher concentration will be pumped to the inside of the sperm, causing a very reproducible swelling of the sperm tails.

The manuscript of Jeyendran et al. provides the methodology used to establish the subnormal values and the gray zone for detecting subnormal HOS test scores [5]. Our methodology, based on Jeyendran et al.'s modification of the bovine test for human sperm, is presented in Table 1 [5].

The test was modified for human sperm by Jeyendran et al. [5]. They determined that sperm with less than 50% tail swelling was abnormal, with the possibility that even 50–59% (gray zone) may also predict male subfertility. The test is very inexpensive, easy to perform, and, in contrast to other semen parameters, is stable over short time periods [6]. Whereas

the frequency of low HOS test scores was only 5% in men ≤29, it was 5-fold higher in men ≥age 50 [7]. The method for performing the HOS test is seen in Table 1. Figure 1 illustrates the different type of HOS patterns.

Table 1. Hypo-osmotic swelling test procedure.

Semen sample is collected via masturbation into a sterile container or laboratory condom after a 2–3-day period of abstinence (see specimen collection)

The materials and equipment needed are as follows:

1. HOS reagent (hypotonic Sperm Wash Medium with HEPES)
2. 12.75 mm glass tubes
3. 1, 5, or 10 mL serological pipet
4. 37 °C heat block
5. Phase Contrast Microscope
6. Glass slides
7. Disposable transfer pipets
8. Cover slip
9. 50–200 µL pipette (Finnpipette/MLA)
10. 50 mL flask
11. Sperm Wash Medium with HEPES
12. DI water

The following steps are needed to prepare the reagent:

1. Equal parts sperm wash medium with HEPES and DI water
2. Mix by inverting
3. Label with name of reagent, lot number, expiration date, and storage information
4. Store refrigerated (2–8 °C) until expiration date, or for 12 months stored at less than 0 °C

For quality control each lot of HOS reagent should be checked against the current lot of HOS reagent. This is achieved by running a patient sample on both lot numbers and checking that the results are ±3 difference.

A viability test for sperm should always be conducted in conjunction with the HOS test. The HOS score cannot exceed the percentage of viable sperm.

The procedure for the HOS test is as follows:

1. Place 1 mL of HOS reagent into a glass 12 × 75 mm test tube
2. Put tube into 37 °C dry heat block and allow it to come to temperature
3. Add 100 µL of well-mixed liquefied semen to the tube and mix well with a transfer pipet. Label with patient's initials and notate the time the test was started.
4. Incubate the mixture in a 37 °C heat block for at least 30 min and no more than 3–4 h.
5. After incubation, mix the solution using a transfer pipet, then place a drop onto a glass slide and cover slip.
6. Read under 40× using a phase contrast microscope.
7. Examine 100 sperm cells on slide (in duplicate) for any signs of swelling (see interpretation chart). Report the HOS score minus the initial coiled tails as final HOS results.

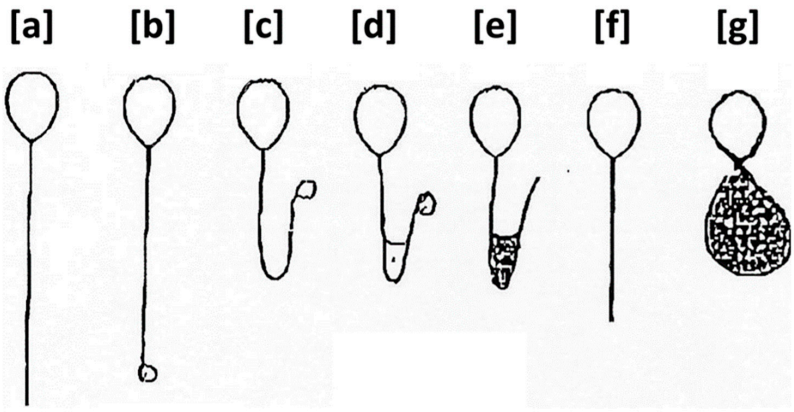

Figure 1. Schematic representation of typical morphological changes of human spermatozoa subjected to hypoosmotic stress: (**a**) = no change, (**b**)–(**g**) = various types of tail changes. Tail region showing swelling is indicated by hatched area.

Interpretation:
Normal HOS score is greater than or equal to 50% swelling
Abnormal HOS score is less than 50%

2. Reduced Fecundity Associated with Low HOS Test Scores

2.1. Evaluation of the HOS Test as a Predictor of Subfertility in Males with Normal vs. Subnormal Motile Densities

A study was performed in which 135 couples, who experienced at least 1 year of infertility, were retrospectively evaluated. In this study, a female factor of infertility was identified and was thought to be correctable by treatment. A subanalysis did not find a predictive value for the gray zone of 50–59%, so they were included with males with normal HOS test scores [8]. The finding that the gray zone of the HOS test does not predict male subfertility was confirmed in later studies [9]. The data were not only stratified according to HOS test scores <50% vs. ≥50%, but according to whether the motile density was normal vs. subnormal.

The data confirmed other studies, which previously mentioned that sperm with subnormal motile densities are not very good predictors of male subfertility. The 8-month pregnancy rates with just natural intercourse were similar in couples for whom the motile densities were subnormal vs. normal, as long as the HOS test was normal. However, when the HOS test score was <50% no males achieved a pregnancy when the motile density was subnormal. However, quite interestingly, no males with normal motile densities achieved a pregnancy within 8 months when the HOS test score was <50% [8]. The gray zone score of 50–59% did not seem to predict subfertility [9].

Based on the assumption at that time (30 years ago) that the main function of the sperm was to fertilize the eggs and create embryos, the most likely explanation at that time for the failure to conceive with low HOS test scores was that the abnormal functional integrity of the sperm membrane caused fertilization failure.

2.2. Studies Questioning the Importance of the Subnormal HOS Test in Predicting Male Subfertility

One of the main ways to determine the fertility potential of sperm is to determine, following IVF, the fertilization rate and the subsequent quality of day 3 embryos or day 5 blastocysts.

There were some early studies that found a slightly lower fertilization rate with lower HOS test scores following IVF-ET. However, they looked at the data in a different way. Liu et al. found the HOS test score to average 65% in patients with <50% fertilization vs. 77% in those with >50% fertilization [10]. Tukahashi et al. found a stronger correlation with IVF fertilization rates than other semen parameters [11].

However, a succession of subsequent studies failed to find any predictability of subnormal HOS test scores in fertilization rates [12–15]. Interestingly, none of these four studies finding no predictability of the low HOS test score on fertilization rates included their pregnancy rates [12–15]. The question arises, were the pregnancy rates omitted because of embarrassment over low pregnancy rates from very good IVF centers? Is it possible that low HOS test scores cause infertility not by fertilization failure, but by causing embryos that appear normal morphologically, yet severely lack implantation potential?

2.3. Evidence That, While Sperm with Low HOS Test Scores Do Not Adversely Affect Fertilization and Embryo Development, Successful Implantation of the Embryo Is Markedly Reduced

Pregnancy outcome and fertilization rates were compared in 27 matched pairs of couples, where one male partner had a low HOS test, and the other one was normal [16]. There were no differences found in fertilization rates comparing low vs. normal HOS test scores. However, there was a marked difference in both clinical and live-delivered pregnancy rates. In 27 matched pairs, the clinical and live-delivered pregnancy rates were 25.9% and 18.5%, respectively, with HOS test scores ≥50, vs. 3.7% and 3.7% for HOS test scores <50% [16].

Another study evaluated 22 donor egg recipient pairs sharing one cohort of oocytes, but where one male partner had an HOS test score <50%, and the other partner, an HOS score ≥50% [17]. Only males with normal other semen parameters were included.

The mean fertilization rates were similar in those with subnormal vs. normal HOS test scores—67.2% vs. 60.9%. A similar number of embryos were transferred (3.3 vs. 3.2). Embryo morphology was also similar. The clinical pregnancy rates for males with HOS test scores ≥50% was 50%, vs. 0% for HOS <50% [17]. This study confirms the previous finding that low HOS scores do not impair fertilization or embryo development, but prevents the embryo from implanting.

A study was performed evaluating the relative adverse effect of low motile density vs. abnormal morphology, using strict criteria, and the HOS test, on the outcome following IVF-ET [18]. With conventional oocyte insemination, the clinical pregnancy rates with normal sperm vs. sperm with low motile densities vs. sperm with strict morphology ≤4% were 27.5%, 15.7%, and 38.9%, respectively. In contrast, the clinical pregnancy rate was 0% with low HOS test scores [18]. The live-delivered pregnancy rates were 21.8%, 15.7%, 38.9%, and zero %, respectively [18].

3. The Association of Low HOS Test Scores and Other Subnormal Semen Parameters

The Sperm Viability Test (Sometimes Called the Vitality Test) vs. the HOS Test

The sperm viability (vitality test) evaluates defects in the structural integrity of the sperm membrane. Sperm with a structural defect in the sperm membrane allow penetration of supravital dye, and are usually non-viable sperm [19]. With the structural defect, water entering the rift in the membrane should also have exit easily, and thus not cause tail swelling. However, if entry is easier than exit, there may be tail swelling. Nevertheless, the assumption would be that the majority of males with low HOS test scores with a functional defect in the sperm membrane, but with a membrane that is structurally intact, should still be able to preclude entry of a supravital stain. A study performing the sperm vitality test on sperm with low HOS test scores from 361 males found only 45 (12.5%) with subnormal sperm vitality tests using eosin as the dye (normal established as >50% of the sperm tested excluding the dye) [20].

There are many fertility specialists who think that the HOS test and sperm vitality are synonymous, or at least test the same thing. In fact, this is not true [21]. As expected, there were no males with subnormal vitality tests who had normal HOS test scores [20].

A study was performed in order to determine if the relative discrepancy between vitality and HOS test scores could predict outcome following IVF-ET, if the HOS test score was >50% [22]. There were 58 patients in the study, and the discrepancy scores ranged from 2.3 to 40.4%. The data were stratified into four quartiles. There was no obvious trend to show that higher discrepancies impair pregnancy rates following conventional oocyte insemination [22].

Some studies attempted to determine if the frequency of low HOS test scores correlate with any abnormality in a given semen parameter. Over a 10-year time period, males were identified who had a single abnormality in a semen parameter, including low concentration ($<20 \times 10^6$/mL), progressive motility <50%, morphology using strict criteria <4%, and the presence of >50% antisperm antibodies [23]. Low HOS tests were observed in <5% of these single semen abnormalities, with the exception of low progressive motility, where it occurred 14.2% of the time [23]. There did seem to be a greater frequency of HOS test scores <50% with a decline in the percentage of progressive motile sperm. Males with 40–49% motility had abnormal HOS test scores in 10% of males vs. 33% in males with 0–19.9% motility [23].

Males with high DNA fragmentation index (DFI) scores are less likely to achieve a pregnancy leading to a live baby [24–26]. A study conducted in order to determine the relationship of DFI and abnormal HOS test scores found only 5 of 87 (5.7%) specimens with normal DFI had HOS test scores <50%, compared to 8 of 31 (25.8%) with DFI >30%. This is compared to 11 of 74 (14.8%) with DFI 15–30% [27].

For 24 males with HOS test scores <50%, a DFI over 30% was found in 8 males (33.3%) vs. only 23 of 168 specimens (13.7%) with normal HOS tests with DFI >30% [27].

One question arises as to whether abnormalities in the acrosome or post-acrosomal sheath could be involved somehow in the etiology of HOS test abnormalities. A priori, this would seem unlikely, since the sperm with low HOS test scores are capable of fertilizing of oocytes without performing ICSI. Sperm with globozoospermia (round-headed sperm) have a complete absence of the acrosome and post-acrosomal sheath and, without ICSI, would not fertilize oocytes [28]. The HOS test was evaluated in two men with 100% globozoospermia. The test scores were 72% and 69% (perfectly normal) [29].

4. Hypothesized Etiology of Infertility from Sperm with Low HOS Test Scores

4.1. Hypothesis as to Mechanism of How the Abnormal HOS Test Causes Infertility

The fact that the conventional insemination of oocytes by sperm with low HOS test scores, with the transfer of normal embryos, rarely leads to a live delivery suggests that this abnormality must cause embryo implantation defects [17,30]. The sperm that attach to the zona pellucida remain attached after fertilization of the egg, even after the one sperm that penetrates the zona pellucida and causes fertilization of the egg has occurred. The zona pellucida becomes incorporated into the embryo membrane, which would include the sperm that are attached. Possibly, there is some toxic factor added to the sperm as it passes through the ejaculatory ducts that causes the functional impairment of the sperm membrane. This toxic factor, in turn, will cause the embryo to have a functional impairment of the embryo membrane, which precludes proper attachment of the blastocyst to the endometrium, or precludes its penetration into the endometrium. The association with advancing age of the male may be related to an increase in this toxic factor by aging ejaculatory ducts [31]. If this hypothesis is true, then confirmation of the implantation defect could be obviated by avoiding contact of the zona pellucida with sperm by injecting one sperm directly into the oocyte, i.e., ICSI.

4.2. IVF with ICSI to Correct Infertility Associated with Low HOS Test Scores

The first reported case of attempting to use IVF with ICSI for sperm with low HOS test scores was published in a pilot series of four cases in 1997 [32]. Live-delivered pregnancies were achieved in two of the four IVF-ICSI cycles with fresh ET [32].

Subsequently, the effect of IVF with ICSI was performed on the female partner of 53 couples where the male partners had an HOS test score <50%, leading to chemical pregnancy in 26 (49.0% per transfer) and viable pregnancy (past 12 weeks) in 24 women (45.3%), with an implantation rate of 27.1% [33]. This provided convincing evidence that not only was IVF with ICSI an effective treatment for couples with infertility related to male partners having sperm with low HOS test scores, but supported the hypothesis that the cause of the infertility is not the sperm that fertilizes the egg, but the sperm attached to the zona pellucida.

Though a 45% ongoing pregnancy rate following IVF with ICSI for low HOS test scores indicates that ICSI can overcome this defect in a high percentage of patients, the question arises as to whether this defect is completely correctable by IVF with ICSI. To help answer this question, a comparison was made in couple pairs sharing one pool of oocytes, but where one male had an HOS test score >50% and the other <50% [34]. The results of 73 such pairs showed a live-delivered pregnancy rate of 49.0% with low HOS vs. 50.0% with normal HOS test scores [34].

Other studies evaluated whether the addition of another sperm abnormality in addition to a low HOS test score could adversely affect the success with IVF with ICSI. The results showed that, whereas the confounding addition of low normal morphology using strict criteria resulted in a viable pregnancy rate of 45.3%, the viable rate of those with a low HOS test score and normal morphology was 34.3% [35].

The confounding effect of poor motility added to an HOS abnormality was also evaluated [36]. There did not seem to be any adverse confounding effect of poor percent

motility, or even a trend in that direction, using sperm from males that also have HOS test scores <50% [36].

The same IVF center responsible for the previous studies of the confounding effect of additional abnormalities in specific semen parameters added to low HOS test scores following IVF with ICSI re-evaluated these additional factors in males with low HOS test scores in the last 10 years. These data were presented at the 2021 meeting of the American Society for Reproductive Endocrinology [37]. The data are presented in Table 1. Again, low motile density, low morphology using strict criteria, and even low percent motility, demonstrated no adverse effect on live-delivered pregnancy rates following IVF with ICSI in males with low HOS test scores (Table 2).

Table 2. Pregnancy outcomes following IVF-ET and ICSI and the confounding effect of other abnormal semen parameters.

	Motile Density <10 × 10^6/mL	**Motile Density ≥10 × 10^6/mL**	**Normal Morphology Strict Criteria <5%**	**Normal Morphology Strict Criteria ≥5%**	**% Motility <30%**	**% Motility ≥30%**
# transfers	106	76	95	74	71	111
Avg. age of female partners	38.6	40.6	39.1	40.0	39.1	39.8
% clinical preg. rate/transfer	32.1%	25.0%	37.9%	21.6%	25.4%	31.5%
% live-delivered preg. rate/transfer	27.4% (*n* = 29)	15.8% (*n* = 12)	31.6% (*n* = 30)	13.5% (*n* = 10)	22.5% (*n* = 16)	22.5% (*n* = 25)
Avg. HOST score	42.4%	43.5%	46.1%	42.9%	44.3%	42.6%

4.3. Consequences of Most IVF Centers Not Performing the HOS Test

The introduction of ICSI, by Palermo et al., markedly broadened the horizons of couples achieving pregnancies despite extremely low concentration, or motility, absence of acrosomes, and even testicular sperm in males with both obstructive and non-obstructive azoospermia [38]. However, ICSI has some disadvantages [39]. Even statistics for the Society for Assisted Reproductive Technology (SART) show that ICSI can lower pregnancy rates compared to conventional oocyte insemination, in part, by not allowing the zona pellucida to choose the "right" sperm, and possibly by some damage to the oocyte in performing the ICSI procedure. Thus, many IVF centers will fertilize oocytes using the conventional insemination technique if the standard semen analysis is normal. One study evaluating males ≤ age 39 found a low HOS test score in 206 of 1663 (12.3) subjects with otherwise normal semen parameters, and 44 of 330 (13.4%) in males aged 40–42 [40]. Thus, almost 13% of couples may use the wrong method of oocyte insemination by not performing the HOS test, i.e., where ICSI should be performed despite otherwise normal semen parameters [40].

4.4. Enzymatic Treatment of Sperm with Low HOS Test Scores to Improve Pregnancy Rates following IUI

The presence of antisperm antibodies bound to sperm is well known to be an infertility factor, especially when present on a high percentage of the sperm [41]. Antisperm antibodies are proteins. Denaturing this antisperm antibody protein by treating the sperm with the protein digestive enzyme chymotrypsin-galactose prior to IUI markedly improved pregnancy rates following IUI [42].

The possibility was considered that the hypothesized toxic factor added to the sperm as they traverse the ejaculatory ducts could be proteinaceous in nature. Though prior studies found no association of the presence of antisperm antibodies and low HOS test scores, researchers decided to try the same treatment of sperm with low HOS test scores with the protein digestive enzyme that was effective in treating sperm with antisperm antibodies [42].

The first study evaluating the efficacy of chymotrypsin pretreatment of sperm with low HOS test scores prior to IUI was conducted in 1997 [32]. Eight patients had IUI with chymotrypsin-treated sperm in 12 cycles. Four of these patients conceived (50% of the patients, 33.3% per cycle) [32].

Subsequently, a larger matched controlled study was performed comparing the outcome in 155 IUI cycles for sperm with low HOS scores vs. 155 IUI cycles for other male factor issues aside from an HOS abnormality [43]. The live-delivered pregnancy rates were 34% for abnormal HOS scores vs. 29% for other male factor problems [43]. It should be noted that, with low HOS test scores, it is important to remind the patients to avoid unprotected intercourse, since sperm reaching the zona pellucida through natural intercourse would still prevent the embryo from implanting [43]. This is important because another study found that the pregnancy rate following IUI with chymotrypsin-treated sperm to be only 9.7% (9/93) for two IUI cycles vs. 33% (142/431) for IVF with ICSI [44]. However, it was subsequently found that the patients treated with IUI were not advised to prevent unprotected intercourse. Nevertheless, though IVF with ICSI will result in higher pregnancy rates per cycle than IUI with chymotrypsin-treated sperm, the latter is a lot less expensive [44].

Though treating the sperm with chymotrypsin prior to IUI is not difficult, for infertility centers that do not have the reagents or experience, another option is to purposely freeze the sperm, then thaw, and perform IUI, since this hypothesized toxic protein may be cryolabile [45]. This option was considered when it was realized that achieving a live delivery with IVF following conventional oocyte insemination is extremely rare, yet live deliveries have been achieved following the transfer of cryopreserved embryos that had been fertilized by conventional oocyte insemination [46].

Though intrauterine implantation with successful live delivery is very rare with natural conception in the female partners of males with low HOS test scores, ectopic pregnancies have been found [47].

4.5. Other Possible Ways to Negate the Adverse Effect on Female Fecundity Related to Male Partners' Sperm with Subnormal HOS Tests

Some studies have suggested that deficiencies of selenium and carnitine may be associated with male infertility [48–50]. One study found an association with decreased selenium and carnitine levels and low HOS test scores [51]. This allows speculation that possibly, in some males, supplementing these nutrients could improve subnormal HOS test scores, and thus improve the fertility potential of the sperm. No clinical studies in humans have evaluated this treatment option as yet.

Another study found that the incubation of sperm with pentoxifylline and coenzyme Q10 in males with oligoasthenoteratozoospermia (OAT) could not only improve DNA fragmentation, sperm apoptosis, mitochondrial activity, and the sperm chromatin dispersion test, but also the HOS test [52]. It would be interesting to determine if incubation with these two agents could improve HOS test scores even in males with isolated defects in the functional integrity of the sperm membrane, and, even more importantly, whether such treatment could improve fertility following IUI, or cause a further increase in success following IUI when combined with chymotrypsin.

It should be re-emphasized that these methods should be used to treat sperm with a functional defect in the sperm membrane, rather than those with a structural defect. This distinction is important because the first two editions of the World Health Organization laboratory manual described the determination of live sperm using a dye exclusion method as a sperm viability test. However, in subsequent editions, it was classified as a vitality test. No explanations were provided as to the reason for this change. Vitality means that the sperm are merely alive. As recently stated by Hecht and Jeyendran (Dr. Jeyendran is one of the pioneers of the HOS test especially adapted for humans) stated that "the HOS test, which assesses the functional integrity of the human sperm membrane, was placed in the same category as the dye exclusion test. Although the two terms might

seem synonymous the term "vitality" merely means "alive" whereas "viability" assesses qualities or physiological function of a living entity. After comparing the morphological, physiological and clinical findings obtained from dye exclusion testing vs. the HOS test, we concluded that the HOS test should be classified as a viability test, not merely as a vitality test" [21]. Whereas males with AOT would be likely to have both abnormal dye exclusion tests and low HOS test scores (a structural defect in the sperm membrane will lead to a functional defect as well), males with otherwise normal semen parameters are very unlikely to have an abnormal dye exclusion test, yet a low HOS test score is very possible.

In the experience of these authors, whose infertile patient population consists generally of treatment-refractory patients, and thus have been previously evaluated in infertility centers in all parts of the United States and other areas of the world, we find that not only is the HOS test not routinely evaluated when performing semen analysis, but this test is not even offered by most infertility practices. However, sometimes a patient from a large geographical distance informs us during a telehealth consultation that their infertility specialist can perform the HOS test, but in fact, evaluates the dye exclusion test. It is very important to make it clear that the two tests are not synonymous, and the HOS test likely has much more clinical value because it can detect a male fertility problem despite an otherwise normal-appearing semen analysis.

5. Improving Outcome of IVF-ET with ICSI by Selecting Sperm with Normal HOS Changes

Selecting Sperm That Are Non-Motile for IVF with ICSI

Motile sperm are required for conception through natural intercourse or IUI. Non-motile sperm can also achieve fertilization and subsequent pregnancies if IVF with ICSI is performed. However, it is required that the sperm injected into the egg is at least alive. This also applies to sperm extracted from the testes, which frequently are non-motile.

Ejaculated sperm that are non-motile may be related to defects in the ciliary body, e.g., Kartagener's syndrome, abnormalities in oxidative stress, or even infection [53]. HOS has been used to select viable sperm in males with necrospermia to fertilize eggs, and live delivery has been achieved [54–57].

Though necrospermia is rare in ejaculated specimens, it is very commonly found in sperm extracted from the testes. As early as 1997, studies were published about successful fertilization with sperm extracted from the testes [58–61]. Nevertheless, studies found that ICSI with non-motile sperm provided embryos of lesser quality, resulting in lower live-delivered pregnancy rates compared to IVF with ICSI for other sperm abnormalities [61].

Selecting sperm through HOS changes is one method by which to identify live sperm without exposing the sperm to harmful chemicals. The tail swelling presents some challenges to placing the sperm in the ICSI needles, but certain modifications of the ICSI technique that allow easier sperm pick-up have been made and reviewed by Nordhoff et al. [62]. Sallam et al. found improved fertilization rates using HOS selection from 30.3 to 44.0% when fresh testicular sperm was used and from 25.7 to 42.7% when frozen testicular sperm was used [63]. Twin pregnancies were reported using the HOS method to select live sperm for ICSI in Kartagener's syndrome [64,65].

6. Possible Significance of HOS Subclasses Are Determined by Different Tail-Swelling Patterns

WHO Classification of HOS Patterns and Association with Sperm Abnormalities

In the WHO Laboratory Manual for Examination of Processing of Human Sperm, starting with the 3rd Edition in 1992, there were seven different patterns described. In 2017, Roven et al. described a modification of the B pattern with smaller coiling of the distal tail and referenced this new pattern as B+ [66]. The B+ pattern shows a ratio of the length of the flagellum to the diameter of the flagellum distal loop that is greater than 20 [66]. Thus, the eight patterns are referred to as A, B, B+, C, D/E, F, and G. Type A shows absolutely no swelling changes at all.

One study evaluated the TUNEL in order to detect DNA fragmentation in sperm with low HOS test scores and found that type A had the highest DNA fragmentation rates (34%), and the second highest was pattern G, with 15% [67]. Another study also found patterns A and G to have the highest DNA fragmentation, but also pattern F [68]. Another study agreed with Stanger et al. that the D pattern had the lowest level of DNA fragmentation, but also the lowest levels of DNA decondensation and phosphatidylserine externalization [69].

Bloch et al. found that, by evaluating the association of these HOS test patterns, including DNA fragmentation, DNA decondensation, and nuclear architecture, types A and G are the sperm with the poorest quality, while B and B+ are the sperm with the highest quality [67].

Studies have been performed linking certain HOS patterns of swelling to aneuploidy. One study found that spermatozoa with patterns B, C, and D/E were 17-fold less likely to have aneuploidy than unselected sperm [70].

Thus, the use of selecting sperm with HOS changes, especially obviously avoiding patterns A or G, seems to have potential to improve the success rates of IVF with ICSI. It would seem prudent to compare the use of ICSI for sperm with low HOS test scores with unselected sperm, vs. sperm selected by HOS changes. Similarly, it seems logical, based on previous studies, to use HOS selection for ICSI from males with chromosomal structural rearrangement leading to miscarriage in the female partner, or even consider it for males of an advanced reproductive age, even if the HOS test is normal. The caveat is to be sure that the extra requirements of collecting sperm with HOS changes into the ICSI needle does not negate the benefits of selection by HOS.

There is, at present, great interest in improving IVF-ICSI outcomes by finding the "right" sperm to inject. As mentioned, selection by HOS changes from immotile sperm can achieve fertilization and live deliveries, but the efficacy of this procedure must be compared to other methods, including the sperm tail flexibility test, especially for fresh sperm [71]. One could also select sperm that were immotile by inducing sperm motility. Methylxanthine derivatives, e.g., pentoxifylline and theophylline, have been used [72,73]. Laser-assisted immotile sperm selection (LAISS) has also been used, based on the principle that a single shot of 129 macro/l for 1.2 MS directed to the tip of the flagellum causes a curling or coiling of the tail of a live but immotile sperm. Aktan et al. found this technique comparable to the HOS technique in identifying live sperm (about 22% for each test) [74]. Gerber et al. also used this technique in order to establish a live delivery following IVF-ICSI in a male with primary cilia dyskinesia, as observed in males with Kartagener's syndrome [75]. LAISS combined with the HOS test may be valuable in cases where no or a very low percentage of sperm show HOS changes [75]. Other methods of sperm selection with potential to improve success rates following IVF with ICSI have been summarized by Simopoulou et al. [71].

7. Final Comments

The HOS test is very inexpensive, costing less than one dollar in materials, and is easy to perform. Unfortunately, probably due to a lack of commercial interest, plus confusion between the HOS test and the sperm vitality test, and several publications questioning the importance of this test because it does not predict fertilization failure following the conventional fertilization of eggs, it is the experience of our infertility center (which, for many years, has treated patients who failed to conceive in other infertility centers), that the HOS test is rarely ever performed in the large majority of medical centers treating infertility.

The failure to identify this problem can lead to some patients having to spend needless money, and go through years of frustration, because of the failure to evaluate this abnormality, which is relatively common in males of advanced age. Since, frequently, women whose male partners are of advanced reproductive age may also be somewhat older, this could lead to the suggestion of the use of extremely expensive donor eggs. What is even more troublesome is that, unless the sperm specimen is slightly abnormal, leading to the use of ICSI, the couple may become severely financially depleted because fresh donor eggs fertil-

ized by conventional insemination of sperm with a low HOS test will not lead to successful pregnancies, despite the transfer of morphologically normal embryos. Furthermore, simple treatment of the sperm with chymotrypsin, which is neither complicated nor expensive, can allow pregnancies by IUI, which may be the only option for some couples without either very good insurance or the financial means to undergo IVF-ET.

Based on the finding of extremely low (in most studies no) pregnancy rates despite the transfer of morphologically normal-appearing day 3 embryos, yet normal live-delivered pregnancy rates by performing ICSI, it is clear that there is good evidence to suggest that sperm with low HOS test scores somehow cause embryo implantation defects. The exact mechanism of how it causes the implantation defect is not known; however, since the HOS defect causes a functional impairment of the sperm membrane, sperm that attach to the zona pellucida remain attached, and the embryo membrane is incorporated into the embryo membrane, one speculation of the mechanism could be that the attached sperm lead to a functional impairment of the embryo membrane, thus preventing the implantation of the embryo. Nevertheless, there certainly could be other explanations than this hypothesis. It is hoped that this manuscript will generate interest in other researchers of IVF and male factors to consider other mechanisms, which, in turn, could lead to other less expensive fertility treatments aside from IVF with ICSI, especially if IUI with chymotrypsin-treated sperm does not lead to a live delivery. Of course, it is important for this review to stimulate interest in other fertility centers to not only perform this test, but also to conduct similar studies in order to either corroborate or challenge these findings.

The fact that the treatment of sperm with chymotrypsin, i.e., a protein digestive enzyme, can not only improve HOS test scores above 50%, but also lead to live deliveries, is consistent with the hypothesis that a toxic protein added to the sperm may be responsible for causing this defect. Treating sperm with a low HOS test with chymotrypsin prior to conventional oocyte insemination before IVF-ET also achieved live deliveries following embryo transfer [76]. For our population of patients with treatment-refractory infertility, we still prefer a day 3 embryo transfer. However, today many IVF centers prefer the transfer of blastocysts. It should be very interesting to infertility centers willing to perform controlled studies to corroborate or refute the aforementioned conclusions, that conventional oocyte insemination with sperm with subnormal HOS tests leads to embryo implantation defects (and thus subsequent failure to produce a live baby), whether the defect may lead to decreased progression to blastocysts or to blastocysts with decreased quality. If blastocyst formation and morphology were similar, but poor pregnancy rates ensue after embryo transfer, this outcome would support the hypothesis that the presence of this toxic factor on sperm leads to embryo membranes with abnormal implantation potential. However, if the development to blastocysts of good quality is impaired, one may need further studies to evaluate how attachment of the sperm with low HOS tests could impair blastocyst formation. This could lead to other studies evaluating the possibility that sperm with defects other than low HOS test scores may inhibit a day 3 embryo from progressing normally. Even if blastocyst formation is normal, it is hoped that this manuscript will encourage future studies to evaluate the role of sperm in preventing embryo implantation, even if the HOS test is normal.

8. Limitations of this Manuscript

The sinequanon of a research study is a properly designed prospective randomized controlled study and, if possible, even multicentered. The basis for the claim that a subnormal HOS test score leads to embryo implantation defects has included prospective matched controlled studies [6,16,48] and prospective observational case studies [33,36], but no prospective controlled studies. Though studies from other centers finding no predictability of low HOS test scores in causing poor fertilization or embryo development did not include their live-delivered pregnancy rates [12–15], which led to our speculation that possible embarrassment over poor live-delivered pregnancy rates led to failure to include this information, this is pure speculation on our part, and, in fact, may not be

true. Possibly, if this manuscript is read by these authors, and they did indeed observe live deliveries, they may write a letter to the editor of *Reproductive Medicine*, which may be subsequently published.

Though a case report involving the HOS test was published in 2020 [45], most of the publications from our group demonstrating implantation defects due to low HOS scores were published over 10 years ago. A careful literature search failed to find any subsequent studies involving the HOS test from other andrology, or reproductive endocrinology/infertility groups, either refuting or corroborating our findings. Unfortunately, while we considered a prospective controlled study in which couples whose male partner had low HOS test scores as the only sperm abnormality, needing IVF-ET, would be randomly assigned to conventional vs. ICSI as a possible research project for our fellow, the ethics committee rejected the proposal because, based on poor live delivery rates in our previous findings, they stated that, in view of the intensity and expense of the process of IVF, it would not be fair to the patients randomized to conventional insemination, despite the offered financial considerations, due to the likelihood of failure to achieve a live delivery. Nevertheless, it is hoped that this published manuscript may generate interest in another andrology or infertility group, not convinced of the value of the HOS test, to perform such a study.

Though we would likely have a better chance of having a prospective study approved by the ethics committee for comparing sperm with isolated HOS abnormalities treated by chymotrypsin prior to IUI, vs. IUI without enzyme-treated sperm to compare pregnancy rates, we never proposed this study. The large majority of our patients are couples failing to conceive in other infertility practices, and it is not likely they would be happy with being relegated to the control group with our strong beliefs that they would not conceive with untreated sperm. However, this could be a good study for other researchers or clinicians who have doubts about this concept. Furthermore, if we conducted a repeat study, only better this time because of the randomization, there still may be concerns with other infertility specialists that all the data seem to be coming from one infertility center, and thus it would be better for such a prospective study to be carried out by a different infertility center.

Hopefully, corroboration of our findings will not only provide a means for helping other patients with unexplained infertility to conceive, but also lead to other research investigations to look for other sperm factors that can lead to embryo implantation defects, especially in older males.

As Drs. Hecht and Jeyendran point out, the HOS test is not synonymous with the sperm vitality or viability test [21]. Whereas all semen analyses with a structural defect of the sperm membrane, as evidenced by a subnormal viability (vitality) test, would have a low HOS test score, a functional defect of the sperm membrane, as evidenced by a low HOS test score, would not necessarily be associated with a structural defect. In fact, most semen specimens with low HOS tests have normal viability (vitality) scores. Perhaps the recent manuscript by Hecht and Jeyendran, and the present manuscript, will convince the WHO committee to once again include the HOS test as a separate sperm test in their next andrology manual, but, if not, hopefully a prospective controlled study by another group corroborating our data will rightfully restore this test in the next edition.

Finally, it should be noted that our hypothesis that this condition is caused by a toxic protein added to the sperm, possibly by its passage through aging ejaculatory ducts, is just that: a hypothesis based not only on improving HOS scores by treating the sperm with a protein digestive enzyme (i.e., chymotrypsin), but also the establishment of live deliveries [32,43,44]. By reintroducing interest in the HOS test, it is hoped that this manuscript will encourage scientists versed in molecular biology to try to determine if, in fact, they can detect some abnormal molecules on sperm with low HOS tests vs. normal ones, or even determine if sperm from aging males may have some molecules attached that are not present in younger males, and that could possibly cause embryo implantation defects, even if not associated with a low HOS test score.

Finally, in order to help determine if the proposed toxic factor causing low HOS tests and implantation defects is cryolabile, and to possibly mitigate failures of having a live delivery in the proposed prospective study of ICSI for low HOS scores by conventional insemination in the controls, the control group could be divided into 50% having fresh embryo transfer, and the other half having deferred transfer with frozen–thawed embryos.

Author Contributions: J.H.C. designed the study and wrote the majority of the manuscript. D.L.C. and A.B. both gathered the data for the study. D.L.C. and A.B. performed the HOST, helped evaluate the data, and helped in writing the manuscript. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Our agreement with Cooper Medical School of Rowan University is that an IRB is only required for a randomized study, so no IRB was required for any of the studies presented in this manuscript. Nevertheless, all treatments were approved by our internal 5 member ethics committee. Ethics Committee for Cooper Institute for Reproductive Hormonal Disorders, P.C., 18 January 2006.

Informed Consent Statement: Patients whose sperm were treated with chymotrypsin/galactose prior to IUI or IVF signed informed consent approving this experimental treatment.

Data Availability Statement: The data used for Table 2 is recent and available. The data from over 10 years ago may be more difficult to obtain.

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