Is there an association between HOST grades and sperm quality?

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BACKGROUND: Intracytoplasmic sperm injection (ICSI) is the primary treatment for male infertility. However for this procedure, with the exception of visual morphological selection, there is no standardization for sperm selection. Recently, the hypo-osmotic swelling test (HOST) has been proposed to potentially select sperm with intact membranes. The aim of this study is to evaluate the ability of this technique to select functional sperm in terms of apoptosis and morphology, as well as nuclear integrity.

METHODS: A total of 20 semen samples were randomly collected from men who attended the Andrology Unit of the Isfahan Fertility and Infertility Center. Semen samples were washed and exposed to hypotonic conditions, before being fixed and simultaneously assessed for membrane integrity as well as abnormal morphology, DNA fragmentation and protamine deficiency by using Papanicolaou, TUNEL and CMA3 staining techniques, respectively. The remaining semen samples were washed with calcium buffer and stained by Annexin V, then exposed to hypotonic conditions before being assessed for early apoptosis along with membrane integrity.

RESULTS: HOST grade ‘d’, followed by grade ‘c’, showed the highest percentages of healthy sperm, whereas sperm of HOST grade ‘g’ in which anomalies in terms of apoptosis, abnormal head morphology or nuclear immaturity or membrane damage, were most frequently observed in the samples assessed.

CONCLUSIONS: Integration of HOST into the sperm selection procedure may provide a valuable tool for selection of functional sperm required for ICSI. According to this study, insemination of HOST grade ‘g’ sperm should be avoided during ICSI.

Key words: HOST / DNA integrity / protamine / apoptosis / membrane integrity

Introduction

Successful applications of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) in humans were initially introduced by Edwards and Palermo (Palermo et al., 1992; Johnson, 2011). Presently, these techniques account for up to 7% of births in some developed countries (Skakkebæk et al., 2006). During ICSI, despite a limited number of oocytes, the oocytes are inseminated by sperm that have been selected based solely on sperm viability and morphology (Nasr-Esfahani et al., 2008). However, recent studies suggest that a high percentage of sperm selected by viability and normal morphology still present a substantial degree of damaged DNA. Therefore, it is estimated that one out of every two oocytes is likely to be inseminated by a spermatozoon that has damaged DNA (Avendaño et al., 2009). Considering the detrimental effect of damaged DNA on reproductive outcomes, novel sperm selection procedures have been proposed to circumvent the possibility of inseminating DNA-damaged sperm. These sperm selection procedures have been based on different sperm characteristics (for further details, see reviews by Henkel, 2012; Nasr-Esfahani et al., 2011; Said and Land, 2011).

Recently, the hypo-osmotic swelling test (HOST) has been proposed to have the potential for sperm selection for ICSI (Stanger et al., 2010). Jeyendran et al. (1984) have introduced HOST as a sperm functional test and have proposed that since this test assesses membrane integrity, which is important in sperm metabolism, capacitation, acrosome reaction and binding of the sperm to the oocyte surface, it might be considered a useful complementary test for assessing male fertility.

The basis of the HOST is the semi-permeability of the intact cell membrane, which induces the sperm tail to ‘swell’ under hypo-osmotic conditions due to an influx of water that leads to expanded cell volume (Drevius and Eriksson, 1966; Ahmadi and Ng,
Considering the importance of this test, a large number of studies have evaluated and reported associations between HOST and semen parameters, and also between HOST and fertilization rates and pregnancy outcomes after both IVF and ICSI (Jeyendran et al., 1984, 1992; Smith et al., 1992; Gopalkrishnan et al., 1995; Check et al., 2001; Cinck et al., 2007; Stanger et al., 2010). These observations may, to a certain extent, be explained by the relationship between membrane integrity and the degree of DNA integrity (Aitken et al., 1998). For example, it is well established that high levels of polyunsaturated fatty acids in the human sperm membrane causes it to be susceptible to reduced motility as well as to oxidative stress which can subsequently induce damage to DNA (Twigg et al., 1998; Aitken et al., 2006). In this regard, HOST has been used along with ICSI to increase fertilization and pregnancy outcomes in severely asthenozoospermic individuals (Buckett, 2003).

Stanger et al. evaluated DNA fragmentation by performing the TUNEL assay on sperm with different HOST grades. They reported the presence of a lower degree of DNA fragmentation in grades ‘d’, ‘e’ and ‘f’ compared with grades ‘a’, ‘b’, ‘c’ and ‘g’ (Stanger et al., 2010). Consequently, in addition to assessing DNA fragmentation in sperm of different HOST scores, we have attempted to evaluate other sperm markers such as externalization of phosphatidylserine as a marker of early apoptosis, as well as protamine deficiency as a sperm immaturity marker and sperm morphology. This study determines the percentage and frequencies of these anomalies in sperm of each HOST score, in order to increase the likelihood of choosing healthy sperm for ICSI.

**Materials and Methods**

This study was approved by the Ethics Committees of the Institutional Review Board of Royan Institute and Isfahan Fertility and Infertility Center.

**Experimental design**

A total of 20 semen samples were randomly collected from men who attended the Andrology Unit of the Isfahan Fertility and Infertility Center. Participants signed written informed consent forms. Semen samples were collected by masturbation in sterile containers after 3–4 days of sexual abstinence. Sperm concentration, morphology and motility were examined according to the World Health Organization criteria (WHO, 2010) before the samples were submitted to the HOST procedure. The flow chart of this study is presented in Fig. 1.

**Sperm preparation and HOST procedure**

We washed 75% of each semen sample with Ham’s F-10 that did not contain albumin (N6635; Sigma, St. Louis, MO, USA). Spontaneous sperm tail curling was also determined for each semen sample, but was considered negligible. Next, 100 μl of the washed sample was diluted with 1 ml of warmed 150 ± 5 mOsm hypotonic swelling solution (Ham’s medium diluted with an equal volume of sterile purified H2O) at 37°C for 5 min. Immediately, the percentage of HOST-positive samples and their grades were determined according to WHO criteria (WHO, 2010). The remainder of each washed samples was fixed for evaluation of the percentages of abnormal morphology, DNA fragmentation and protamine deficiency. The remaining 25% of each semen samples was washed with calcium buffer and stained with Annexin V. A total of 100 μl of Annexin V stained samples was immediately exposed to 1 ml of hypo-osmotic medium at 37°C for 5 min, after which the apoptotic status of sperm in different HOST grades was determined.

**Evaluation of the percentage and frequency of each abnormality**

We simultaneously defined sperm tail swelling grades and different sperm abnormalities that included morphology, protamine deficiency, DNA fragmentation, and early markers of apoptosis in at least 200 sperm. The results were presented in two formats:

(A) The percentage of abnormal sperm for each parameter among sperm of each HOST grade (number of abnormal sperm in each grade/total number of sperm assessed in that grade × 100).

(B) The frequency of the sperm which are abnormal for each parameter and for each HOST grade among the total number of sperm assessed (total number of abnormal sperm for each parameter for each HOST grade/total number of sperm assessed).

Of note, the percentage was obtained in one particular HOST category or grade, whereas the frequency was determined according to all HOST grades in each sample.

**Evaluation of DNA fragmentation by TUNEL assay**

We performed the TUNEL assay (terminal dUTP nick-end labeling), with a commercial detection kit (ApopTosis Detection System Fluorescein, Promega, Mannheim, Germany). After the HOST procedure, 20 μl of each sample was fixed by 4% methanol-free formaldehyde, placed on slides for 25 min at 4°C and then assessed by TUNEL according to our previous studies (Khaiavi et al., 2009; Kheirollahi-Kouhestani et al., 2009). For each sample, a minimum of 200 sperm were randomly evaluated to determine the percentage and frequency of DNA fragmentation for each HOST score.

**Evaluation of protamine deficiency by CMA3 staining**

Following the HOST procedure, 20 μl of the sample was fixed in Carnoy’s solution before preparing the slides. Each slide was then covered for 20 min with 100 μl of 0.25 mg/ml CMA3 solution (Sigma) for 20 min. Next, slides were washed with phosphate-buffered saline, mounted and analyzed. A minimum of 200 cells were counted from each slide. Spermatozoa stained bright yellow were considered as protamine deficient (CMA3 positive) and spermatozoa with dull yellow stain were considered to have the normal amount of protamine (CMA3 negative). Simultaneously while assessing the CMA3 staining in each sperm, the HOST grade of the sperm was also determined. Subsequently, the percentage and frequency of protamine deficiency for each HOST score were determined (Nasr-Esfahani et al., 2001).

**Evaluation of external phosphatidyl serine by Annexin V**

External phosphatidyl serine (EPS), an early marker of apoptosis, was assessed using an Annexin V FITC kit (P-116F; IQ Products, Groningen, the Netherlands). For this, 25% of each semen sample was washed with calcium buffer, and 100 μl of the washed sperm was incubated with 5 μl Annexin V FITC in calcium buffer. Subsequently, the HOST procedure was performed on the Annexin V treated sample with immediate analysis using a fluorescent microscope. For each case, a minimum of 200 sperm were simultaneously assessed for Annexin V positivity and the HOST score was determined. Finally, the percentage and frequency of
Annexin-positive sperm for each HOST score were determined (Muratori et al., 2004).

Evaluation of abnormal morphology by Papanicolaou staining

After the HOST procedure, slides were prepared and stained according to WHO criteria (WHO, 2010). For each case, 200 sperm were simultaneously evaluated for head morphology and HOST scores. Sperm head abnormalities were classified as: large or small; tapered pyriform, round or amorphous; vacuolated (more than 2 vacuoles or >20% of the head area occupied by unstained vacuolar areas); small or large acrosomal areas (<40 or >70% of the head area); double heads; or any combination of the above. Additionally, the exposure of sperm to hypo-osmotic conditions in this study induced curling of the sperm tails, but other abnormalities were not evaluated.

Finally, the percentage and frequency of sperm with abnormal head morphology for each HOST score was determined.

Statistical analysis

Statistical analysis was performed using the Freidman non-parametric test. If a significant overall difference between groups was noted, pair wise comparisons were then conducted with adjusted α (using Bonferroni correction). Differences were statistically significant at P < 0.05. We used SPSS version 16 to analyze the data.

Results

The sperm concentration in the present study ranged from 7 to 151.3 million/ml (mean: 71.05 ± 8.3 million/ml), while the percentage of motility ranged from 0.5 to 75% (mean: 49.9 ± 4.3%) and abnormal sperm morphology ranged from 74 to 98% (mean: 84.63 ± 1.4%).

Figure 2 shows the distribution of different types or grades of sperm tail swelling in from the HOST procedure according to WHO criteria.

In this study, the mean percentage of positive HOST sperm was 51.1 ± 3.3 and the percentage of negative HOST sperm was 48.9 ± 3.3 in 20 samples. The mean percentages for different grades of sperm tail swelling were: 48.9 ± 3.3 (grade a), 10.0 ± 1.1 (grade b), 5.1 ± 0.9 (grade c), 1.9 ± 0.3 (grade d), 9.0 ± 1.1 (grade e), 3.5 ± 0.6 (grade f) and 19.9 ± 2.0% (grade g).

The mean percentage of sperm with each abnormality among sperm of each HOST grade and the mean frequency of sperm with each abnormality and each HOST grade among the total sperm assessed are presented in Fig. 3.

As seen in Fig. 3, the mean percentages of abnormal head morphology in sperm of each HOST grade were: 85.5 ± 2.4% (grade a), 66.0 ± 5.1% (grade b), 77.8 ± 6.5% (grade c), 45.3 ± 11.3% (grade d), 72.8 ± 3.6% (grade e), 78.1 ± 5.5% (grade f) and 85 ± 3.6% (grade g). Due to low numbers, the mean percentage of abnormal head morphology in grade ‘d’ was not significantly lower than in the other grades. The mean frequencies of sperm with abnormal head morphology and grade ‘a’ to ‘g’ amongst total sperm were 34.9 ± 4.2 (grade a), 9.2 ± 1.2 (grade b), 3.8 ± 0.4 (grade c), 1.2 ± 0.4 (grade d), 9.1 ± 1.1 (grade e), 4.2 ± 0.8 (grade f) and 16.5 ± 2.4 (grade g) (Fig. 3). According to the results, the mean frequency of sperm with morphological anomalies was observed for grade ‘d’, which was significantly lower than for all grades, with the exception of grade ‘f’.

The mean percentages of DNA fragmentation in sperm of each HOST grade were: 16.8 ± 3.0% (grade a), 3.9 ± 1.6% (grade b), 4.8 ± 2.6% (grade c), 5.0 ± 5.0% (grade d), 6.9 ± 2.3% (grade e), 15.4 ± 4.9% (grade f) and 13.0 ± 3.2% (grade g) (Fig. 3). The lowest mean percentage of DNA fragmentation was in grade ‘b’ which was significantly lower than in grades ‘a’ and ‘g’. The mean percentage of DNA fragmentation in grade ‘d’ was also significantly lower than in grade ‘a’. The mean frequencies of sperm with DNA fragmentation and grade ‘a’ to ‘g’ amongst total sperm were: 6.9 ± 1.2 (grade a), 0.4 ± 0.2 (grade b), 0.2 ± 0.1 (grade c), 0.0 ± 0.0 (grade d), 0.5 ± 0.2 (grade e), 0.4 ± 0.1 (grade f) and 4.1 ± 1.5 (grade g), respectively (Fig. 3). The mean frequency of DNA fragmented and grade ‘g’ sperm was significantly higher than this abnormality in grade ‘f’.
grades 'b' to 'f'. However, the lowest mean frequency was observed for grade 'd' DNA fragmented sperm, which was significantly lower than for grades 'a' and 'g'.

Assessment of Annexin V, as an apoptotic marker for the evaluation of EPS, revealed that the mean percentages of apoptotic sperm in each HOST grade were: 52.4 ± 4.0% (grade a), 40.1 ± 7.1% (grade b), 20.7 ± 7.1% (grade c), 5.6 ± 3.8% (grade d), 35.2 ± 5.0% (grade e), 46.0 ± 8.4% (grade f) and 40.6 ± 5.2% (grade g) (Fig. 3). The mean percentage of apoptotic sperm in grade 'd' was significantly lower than in grades 'a', 'e', 'f' and 'g'. The mean frequencies of sperm which were apoptotic and grade 'a' to 'g' amongst total sperm were: 25.5 ± 3.2 (grade a), 1.9 ± 0.3 (grade b), 0.5 ± 0.2 (grade c), 0.1 ± 0.1 (grade d), 4.7 ± 0.8 (grade e), 2.0 ± 0.4 (grade f) and 7.6 ± 1.0 (grade g) (Fig. 3). The mean frequency of grade 'g' apoptotic sperm was substantially higher than for grade 'b', 'c', 'd' or 'f' apoptotic sperm. The lowest mean frequency was observed for grade 'd' apoptotic sperm.

Protamine deficiency, as a nuclear immaturity marker, was assessed by CMA3 staining. The mean percentages of protamine deficiency in sperm of each HOST grade were: 48.2 ± 5.2% (grade a), 44.9 ± 6.3% (grade b), 40.3 ± 8.7% (grade c), 1.8 ± 1.8% (grade d), 39.3 ± 6.4% (grade e), 42.1 ± 6.8% (grade f) and 56.7 ± 5.5% (grade g) (Fig. 3). The mean percentage of protamine deficiency in grade 'd' was significantly lower than in other grades. The mean frequencies of sperm with protamine deficiency and grade 'a' to 'g' amongst total sperm were: 14.0 ± 2.5 (grade a), 6.5 ± 1.1 (grade b), 1.7 ± 0.5 (grade c), 0.1 ± 0.1 (grade d), 8.6 ± 1.8 (grade e), 2.3 ± 0.5 (grade f) and 13.1 ± 1.8 (grade g) (Fig. 3). The mean frequency of protamine deficient and grade 'g' sperm was significantly higher than this abnormality in grades 'c', 'd' and 'f'. The lowest mean sperm frequency was observed for grade 'd' protamine deficient sperm, which was significantly lower than all for other grades.

**Discussion**

The quality of sperm selected for ICSI is believed to be one of the determining factors for successful assisted reproduction. Despite improvements, the basis of sperm preparation and selection during ICSI mainly relies on the procedure initially implemented for IVF (Henkel and Schill, 2003). Considering the potential of ICSI to overcome severe male infertility, the inevitable insemination of DNA-damaged or apoptotic sperm, which have normal appearance and motility, may adversely impact fertilization and subsequent development (Tavalaee et al., 2009; Razavi et al., 2010). To overcome this adverse effect, it has been suggested that sperm should be selected based on functionality rather than solely on morphology and viability, in order to reduce the chances of insemination of DNA-damaged or apoptotic sperm (Henkel, 2012; Nasr-Esfahani et al., 2011).

During the passage through the epididymis, sperm undergo a maturation process, prerequisite for motility, capacitation, the acrosome reaction and the ability to bind to the zona pellucida. This level of maturity is acquired during transition through the luminal environment of the epididymis (Zhou et al., 2008). Defective sperm are usually
Figure 3  Mean percentage and mean frequency of sperm with abnormal head morphology, DNA fragmentation, phosphatidylserine externalization (apoptosis) or protamine deficiency for each HOST grade. Left: Percentage of abnormal sperm for each parameter among sperm of each HOST grade. Right: Frequency of the sperm which are abnormal for each parameter and of each HOST grade among the total number of sperm assessed. Common letters are significantly different at $P < 0.05$ and the absence of a common letter means that the groups are not significantly different.
eliminated through this transition by the process of ubiquitination (Varum et al., 2007; Eskandari-Shahraki et al., 2011). In infertile individuals, defective sperm, including apoptotic and DNA-damaged sperm, may bypass this system and may be found in the ejaculate, similar to the situation which occurs with abortive apoptosis in testes (Sakkas et al., 2003).

Recently, it has been hypothesized that defects in the functional integrity of the sperm membrane, which can be detected by the HOST test, may reduce the potential for fertility by causing implantation failure or increasing the rate of spontaneous miscarriage rather than simple fertilization failure (Bhattacharya, 2010). Thus, it is believed that sperm membrane integrity might reflect the degree of DNA integrity.

The results of this study and the study conducted by Stanger et al. (2010) show that it is possible to select sperm with intact DNA based on the HOST grade. This is contrary to previous studies which suggest all the HOST-positive sperm are suitable for ICSI. The results of this present study suggest that the percentage and frequency of DNA fragmentation is greater for HOST grade ‘g’ sperm, similar to the percentage and frequency of non-viable grade ‘a’ sperm. Therefore, considering that 50% of sperm are viable, the likelihood of selecting grade ‘g’ sperm is around 40% (Fig. 2) in viable or HOST-positive sperm. Considering that 13% of ‘g’ grade sperm have fragmented DNA (Fig. 3), the possibility of selecting damaged sperm merely based on viability or motility is around 5% (40% × 13%). Based on the present results, through HOST scoring one might be able to avoid insemination of ‘g’ grade sperm which contain damaged DNA. This selection would be even more useful for severe male infertility cases where the percentage of sperm with damaged DNA is higher, even for sperm with normal head morphology (Avendaño et al., 2009; Avendaño and Oehninger, 2011). In accordance with these results, Stanger et al. (2010) have also shown that the percentage of sperm with DNA fragmentation is higher in some HOST grades.

According to Figure 3, it is best to select grade ‘d’ sperm. In the absence of this grade, appropriate choices are grades ‘c’ or ‘b’, respectively. The trend observed for DNA fragmentation was also observed when other sperm anomalies such as abnormal head morphology, protamine deficiency and apoptosis were considered. Therefore, it has been suggested that insemination of ‘g’ grade sperm should be avoided and where possible grade ‘d’, followed by grade ‘c’ should be considered for insemination.

Since most of these anomalies are common between one or two HOST grades, they are likely to be related to sperm membrane integrity. It has been stated that the reaction of the Na+/K+ pump towards hypotonic conditions results in tail swelling, known as HOST. In sperm with competent membranes, the functional activity of these pumps leads to minimal tail swelling, while in sperm that have an impaired Na+/K+ pump, complete swelling is observed, which leads to the formation of HOST grade ‘g’ (Peris et al., 2000; Stanger et al., 2010). Liu et al. (2007) have suggested that sperm-specific membrane proteins such as HSPA2 are required for correct assembly and trafficking of these pumps. HSPA2 is also required for proper chromatin packaging and histone/protamine exchanges. Dysfunction of HSPA2 causes sperm to be susceptible to DNA damage and impaired membrane integrity (Nasr-Esfahani et al., 2010). The frequency of CMA3-positive (protamine deficient) sperm, as a marker of chromatin integrity, is also highest in grade ‘g’ sperm which increases their susceptibility to DNA damage. Therefore, dysfunction of HSPA2 may be associated with a higher frequency of sperm anomalies and DNA damage in grade ‘g’. However, this point remains to be elucidated.

In addition, Stanger et al. have suggested that the association between HOST and DNA damage was stronger than the link to the presence of vacuoles, therefore arguing that the presence of vacuoles is more of an endpoint visual expression of chromatin degeneration while HOST may be a more precocious indicator. This observation is reinforced by multiple regression analyses that identified relationships between HOST and motility, but not to head or midpiece abnormalities. Therefore, these authors conclude that HOST identifies membrane events rather than terminal nuclear degeneration and might be more appropriate for the selection of sperm required for ICSI (Stanger et al., 2008, 2010).

Recently, Avendaño et al. (2011) have demonstrated that ICSI outcomes do not show a significant relationship with DNA fragmentation in whole samples, but do indicate a significant correlation when normal sperm with fragmented DNA are taken into account. Therefore, they have concluded that ‘the evaluation of DNA integrity in morphologically normal spermatozoa after sperm selection is a better approach to examine sperm DNA fragmentation and any potential impact on ICSI procedure’ (Saleh et al., 2002; Avendaño and Oehninger, 2011). The results of this study show that through HOST sperm selection, it is possible, to a certain extent, to avoid insemination of sperm with normal morphology but with fragmented DNA or other anomalies.

Translocation of phosphatidyl serine from the inner to the outer leaflet of the plasma membrane may be considered as the basis of disturbed membrane integrity and the earliest feature of cells undergoing apoptosis, which can be characterized by Annexin V staining (Zarei-Kheirabadi et al., 2012). Assessment of Annexin V was also observed in a high percentage of grade ‘g’ sperm. Therefore, the selection of sperm based on HOST may prevent insemination of sperm which have just entered the process of apoptosis. Selection using HOST is further supported by the observation that grade ‘d’ and even grade ‘c’ sperm present with minimal externalization of phosphatidyl serine, which suggests that they have intact membranes.

Conclusion
HOST was initially introduced as a simple test for the discrimination of viable from non-viable sperm. This test was later applied for the insemination of viable sperm for ICSI. The results of the present study have revealed the potential of this test to select the best sperm in terms of chromatin and membrane integrity. According to our results, the best sperm are likely to be present in the population of grade ‘d’ sperm, followed by grade ‘c’ sperm, both of which are present at a low frequency. Considering this, the results of this study suggest that insemination of sperm with HOST grade ‘g’ should be avoided, as this grade presents the highest frequency of viable sperm in semen samples.

Authors’ roles
M.H.N.-E. designed and interpreted the results, and contributed to writing, revising, editing and final approval of the manuscript. F.B. performed the experimental analysis and collected the data. M.T. contributed to the methods, interpreted the results and contributed to the
writing of the paper and manuscript revision. A.H.S. supervised student. S.M. who performed the statistical analysis.

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**Conflict of interest**

None declared.

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