Correlation between the Hypoosmotic SwellingT and DNA Fragmentation Assessed by the TUNEL Assay in Asthenozoospermia

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ABSTRACT

Objective: To study the correlation between the hypoosmotic swelling test (HOST) and DNA fragmentation in asthenozoospermia assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL) assay.

Methods: This cross-sectional study was conducted in 27 semen samples obtained from infertile men with asthenozoospermia. Both HOST and TUNEL assay were performed for each sample. The sperm swelling pattern and positive apoptosis staining of individual spermatozoa were evaluated. HOST and TUNEL scores, and the proportion of positive staining in each grade were calculated in each sample.

Results: The results showed a negative correlation between HOST and TUNEL scores ($r = -0.428$, $P = 0.026$). Sperm swelling grade A had a higher incidence of positive apoptosis staining when compared with other grades ($P < 0.01$). There was no statistically significant difference in positive apoptotic staining between other grades; nevertheless, sperm swelling grade D tended to have a lower incidence of positive apoptosis staining.

Conclusion: Based on the results of this study, HOST may be used as an optional test to identify DNA-intact spermatozoa whereby sperm with a grade D swelling pattern should be selected preferentially for intracytoplasmic sperm injection (ICSI), whereas sperm with a grade A swelling pattern should be avoided for ICSI.

Keywords: Hypoosmotic swelling test; TUNEL; asthenozoospermia; male infertility; intracytoplasmic sperm injection (Siriraj Med J 2019;71: 8-13)

INTRODUCTION

Male factor infertility constitutes 30% of infertility causes. The World Health Organization (WHO) defines the lower limit of normal sperm motility as 32% of progressively motile sperm (5th centile, 95% confidence interval [CI]: 31-34) and 40% of total motile sperm (5th centile, 95% CI: 38-42).[1] The prevalence of asthenozoospermia has been reported to be 18.71-24.19%.[2,3] In Thailand, 10.78% of infertile couples are affected by asthenozoospermia.[4] During natural human fertilization, sperm velocity is essential for its transition through the vagina and fallopian tubes, and for the penetration of the cumulus oophorus and zona pellucida. Therefore, sperm motility impacts fertilization rates; i.e., low-velocity sperm are associated with a reduced chance of zona pellucida penetration and thereby fertilization potential.[5-7] Intracytoplasmic sperm

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injection (ICSI) was developed to overcome impaired fertilization as a result of reduced semen parameters, including asthenozoospermia. However, higher DNA fragmentation rates are reported in asthenozoospermia and the introduction of ICSI for asthenozoospermia has promoted the transmission of disintegrated sperm DNA to the resulting offspring, which consequently affects fetal or postnatal development.[8] Many studies have reported a negative correlation between sperm DNA damage and fertilization, implantation and miscarriage rates, in addition to embryo quality and rates of childhood diseases and cancer.[9-14] Thus, it is important to discriminate DNA-intact spermatozoa in asthenozoospermia to improve pregnancy rates and reproductive health outcomes.

DNA integrity can be analyzed by several diagnostic tests, such as terminal deoxynucleotidyl transferase mediated deoxyuridine triphosphate nick end-labeling (TUNEL) assay.[15,16] However, drawbacks associated with the TUNEL assay are that it is a time-consuming technique, which requires specific skills and, most importantly, causes sperm toxicity. Therefore, the adoption of a nontoxic sperm selection test is essential for ICSI. In cases of severe asthenospermia, it is difficult to distinguish viable and nonviable sperm. Since some viable sperm are known to contain DNA-intact components, it is important to be able to select viable sperm for ICSI to optimize fertilization rates.[17]

The hypoosmotic swelling test (HOST) is the most commonly used method for assessing sperm vitality.[1] The basis of HOST relies on the semipermeable nature of the sperm cell membrane, which allows the influx of water when placed in a hypoosmotic solution, and results in the expansion and coiling of the tail.[18] HOST presumes that only cells with intact membranes (live cells) will swell when within a hypotonic solution, which allows easy identification of viable sperm.[1] It is a simple, quick, safe, and cost-effective test. HOST was recently introduced as a test for sperm function because membrane integrity is important for sperm capacitation, the acrosome reaction, and sperm–oocyte binding and penetration. Moreover, HOST has been suggested as an alternative test for DNA integrity. Previous studies have demonstrated a favorable association between HOST and many reproductive outcomes, including fertilization and pregnancy rates.[19-21] Some studies have shown benefits from using HOST for the selection of viable nonmotile spermatozoa.[22,23] Casper et al. found increased fertilization and cleavage rates (43% and 39%, respectively) when HOST was used to select viable sperm compared with when sperm was randomly selected in cases of complete asthenozoospermia (26% and 23%, respectively).[24] Ortega *et al.* also reported improved fertilization rates using HOST in complete asthenozoospermia.[23] In contrast, recent studies have failed to report a benefit of HOST utilization to select sperm on the basis of lower DNA fragmentation levels.[20,21] However, these studies were conducted on semen samples with parameters within the normal range; therefore, they may not be applicable in asthenozoospermia cases.[20,21] There is insufficient data in relation to asthenozoospermia; therefore, we conducted this study to clarify the correlation between HOST outcomes and DNA fragmentation levels in asthenozoospermia as assessed by the TUNEL assay. The secondary objective was to identify the positive apoptosis staining in each sperm-swelling grade.

MATERIALS AND METHODS

This cross-sectional study was approved by the Institutional Review Board of the university hospital and it was conducted in males aged 18 years or older who were attending a university-based infertility unit. All patients who requested a semen analysis and provided written informed consent were screened. Semen samples were collected by masturbation. Twenty-seven patients with asthenozoospermia classified according to WHO 2010 were eligible for this study.[1] Two aliquots of each sample were collected within 90 minutes after semen sample collection and liquefaction. Both aliquots were first prepared for HOST and then fixed for TUNEL assay. The fixed and stained semen sample slides were examined using a phase contrast microscope (BX40; Olympus, Tokyo, Japan). A single interpreter assessed a total of 200 spermatozoa per semen sample twice. The semen parameters, i.e., average HOST score, average TUNEL score, individual spermatozoa HOST grading, individual apoptotic staining, and proportion of positive apoptosis staining in each grade of sperm swelling, were calculated and recorded.

HOST

According to WHO 2010,[1] the hypoosmotic solution was prepared by dissolving 0.375 g of sodium citrate dehydrate and 1.351 g of D-fructose in 100 mL of purified water. An aliquot of 100 mL of semen was mixed with 1 mL of the swelling solution before being incubated at 37°C for 30 minutes. Ten microliters of the mixed sample was then placed on a clean slide and covered with a coverslip. In live sperm with normal membrane function, the hypoosmotic buffer diffused through the membrane into the sperm tail. Under microscopy, live spermatozoa showed various degrees of tail swelling and curling, whereas dead cells exhibited no membrane

changes. All membrane-change patterns were categorized from grades A to G (Fig 1), where grade A corresponds to no tail swelling. The HOST score was obtained from the average percentage of total swollen spermatozoa (excluding the natural swelling before treatment with hypoosmotic buffer). The lower reference limit for normal vitality was 58% (5th centile, 95% CI: 55-63).[1]

TUNEL assay

Both semen slides were fixed after HOST. The TUNEL assay was then performed according to the manufacturer's instructions for ApopTag® Plus Peroxidase *In Situ* Apoptosis Detection Kit (Merck, Kenilworth, NJ, USA). In the assay, spermatozoa with DNA fragmentation stained brown, while sperm containing intact DNA stained green (Fig 2).

Statistical analysis

The semen parameters were presented as mean, median, and standard deviation. The intraclass correlation coefficient was used to evaluate consistency in two time measurements for the HOST and TUNEL scores in each sample. The correlations between HOST and TUNEL scores were analyzed using Pearson correlation and Wilcoxon signed-rank tests. $P < 0.05$ was accepted as statistically significant. Data were analyzed using PASW Statistics (v. 18.0; SPSS Inc., Chicago, IL, USA).

RESULTS

The characteristics of 27 semen samples are shown in Table 1. A fairly negative correlation between HOST and TUNEL scores ($r = -0.428$, $P = 0.026$) was revealed using Pearson correlation (Fig 3). There was high agreement in

The TUNEL score was calculated using the formula:

$$\% \text{ Average positive staining} = \frac{\text{Average of stained apoptotic spermatozoa}}{200} \times 100$$

The proportion of positive apoptosis staining in each grade of sperm swelling was calculated as follows:

$$\text{Positive apoptosis staining (\%)} = \frac{\text{number of positive apoptosis staining in that grade}}{\text{total number of spermatozoa in that grade}} \times 100$$

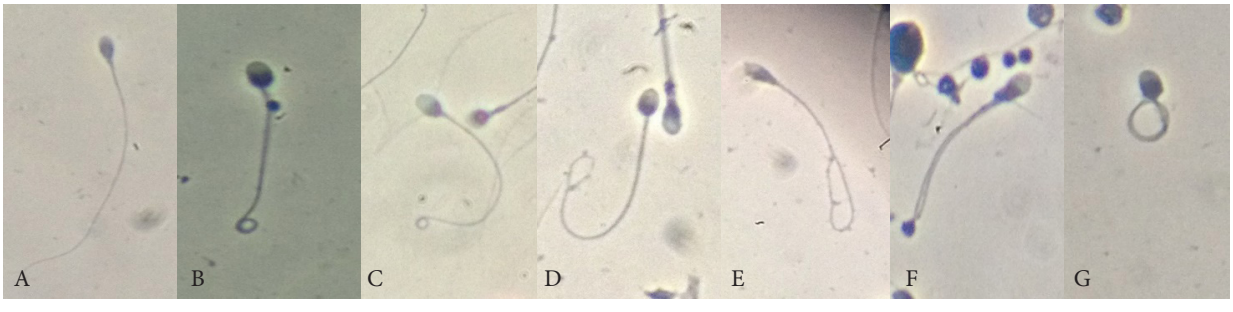

Fig 1. After incubation in hypo-osmotic solution for 30 minutes, every sperm was assessed under phase-contrast microscopy and categorized in 7 groups, from grade A to G as demonstrated in the figure. Grade A showed the sperm with no membrane swelling which indicated a non-viable sperm. Grade B to G showed different grades of tail swelling in viable sperms.

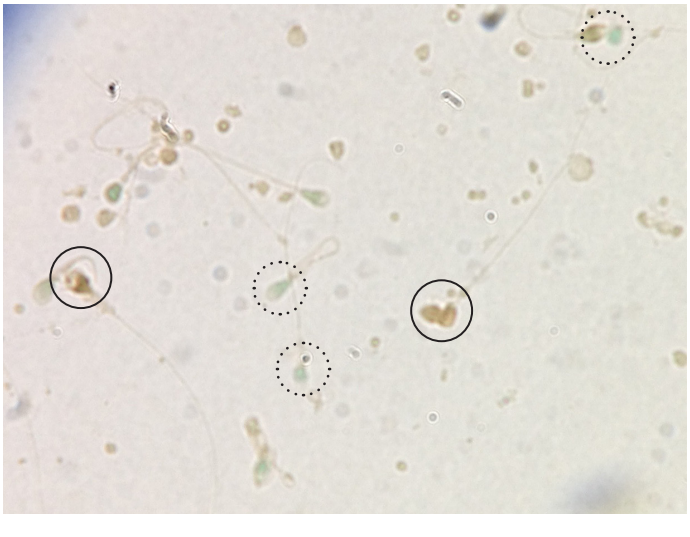

Fig 2. Apoptosis staining under normal light microscopy was demonstrated in the figure.

The positive apoptosis staining was demonstrated in the solid circle.

The negative apoptosis staining was displayed in the circle of dotted line.

TABLE 1. Characteristics of semen parameters (N = 27) were displayed.

Semen parameters	Mean ± SD
pH	7.7 ± 0.3
Volume (mL)	2.5 ± 1.8
Sperm concentration (10^6/mL)	21.4 ± 15.1
Total sperm count (10^6/mL)	51.9 ± 43.7
Progressive motility (%)	24.1 ± 5.1
Vitality (%)	58.9 ± 11.3
Normal morphology (%)	8.9 ± 5.9
Natural sperm swelling (%)	7.2 ± 3.2

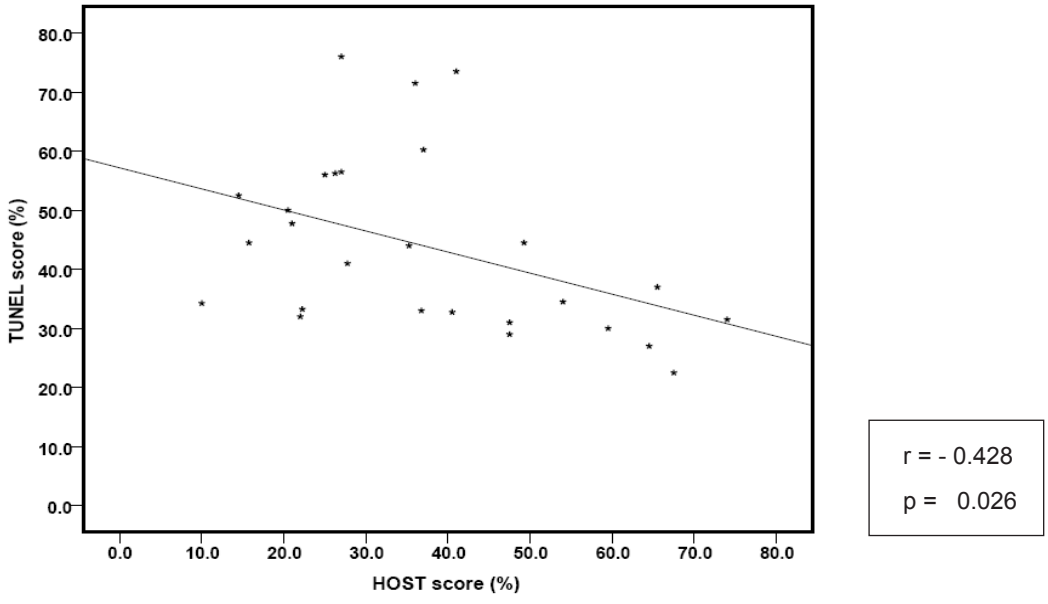

Fig 3. Correlation between HOS TEST score and TUNEL score.

the duplicate test evaluations by the same investigator. The intraclass correlations were 0.97 (95% CI = 0.935-0.986) and 0.952 (95% CI = 0.898-0.978) for the HOST and TUNEL score assessments, respectively.

When the positive staining of TUNEL was compared among the groups with various tail swelling patterns, grade A sperm demonstrated the highest proportion of apoptosis staining ($P < 0.01$). There was no significant difference in the DNA integrity between other grades. Nevertheless, the sperm swelling grade D tended to have a lower proportion of positive apoptosis staining (Fig 4).

DISCUSSION

The quality of sperm is highly associated with the outcome of assisted reproductive technology. The selection of sperm with high DNA integrity is an important step prior to ICSI to optimize the outcome because DNA

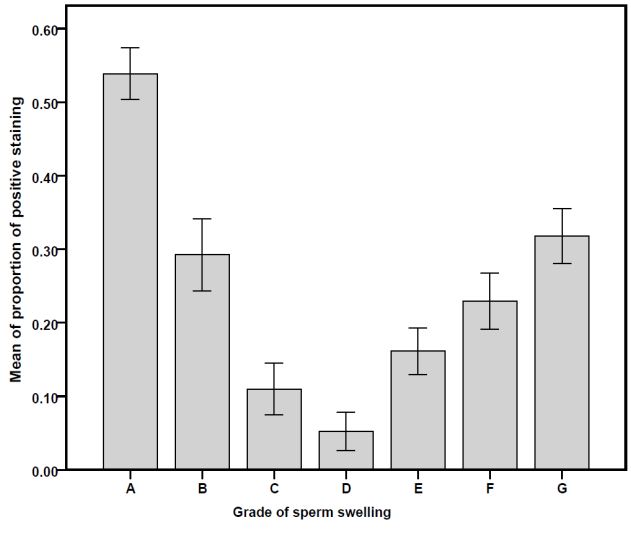

Fig 4. Proportion of positive apoptosis staining in each grade of sperm swelling.

fragmentation is known to affect fertilization rates and embryo development.[25] Assessment based solely on morphology or vitality alone may not be enough because many sperm with normal morphology and motility still contain damaged genetic material, especially in asthenospermia.[8]

Membrane function contributes a key role in various sperm function competencies, i.e., capacitation, the acrosome reaction, and fertilization. Membrane disintegration is suggested to be associated with implantation failure and miscarriage.[26] HOST is a vitality test used to reveal the sperm membrane integrity and it is not only useful in increasing fertilization rates in cases of complete asthenospermia, but can also be used to assess sperm DNA fragmentation.[19,20]

This study demonstrates the relationship between HOST and TUNEL assay in cases of asthenozoospermia and highlights the benefits of using HOST to select sperm with lower levels of DNA fragmentation. The results are consistent with the study by Stanger,[20] which also showed a negative correlation (r = -0.81) between HOST and TUNEL assay outcomes. However, this correlation was stronger than in this study. This discrepancy is likely to be the result of the difference in study populations, with this study enrolling only asthenozoospermic males whose samples are prone to DNA fragmentation.[8]

Stranger reported significantly higher grade A and lower grade D swelling patterns in abnormal semen samples compared with normal samples.[20] However, that study did not directly identify the DNA status in each grade. In this study, the DNA integrity and tail swelling pattern was assessed individually for each sperm; therefore, the DNA integrity pattern for each grade can be calculated. In the DNA fragmentation analysis in each grade of sperm swelling, grade A was found to contain the highest portion of DNA fragmentation, while grade D obtained the lowest percentage. In the remaining viable sperm, healthy DNA was identified mostly in grade C followed by grades E, F, B, and G (Fig 4), which concurs with previous studies that suggested a correlation between tail swelling pattern and DNA damage.[20,21] These results are also consistent with a study conducted in males with normal semen parameters.[27] The HOST or hypotonic resistance test distinguishes the sperm with different capacities in the function of Na^+/K^+ and Na^+/H^+ exchange of the membrane. Sperm that exhibited the minimal swelling patterns, as in grade D, are supposed to contain higher membrane competency along the flagellum.[28] Nevertheless, a high level of swelling signifies impaired Na^+/K^+ ATPase function.[20] The normal HOST-sensitive membrane feature is suggested to be linked with less or no DNA damage.[20] Therefore, this finding suggests that HOST is clinically applicable for selecting better-quality sperm with a grade D swelling pattern for ICSI.

CONCLUSION

There is a fairly negative correlation between HOST outcomes and DNA fragmentation levels in asthenozoospermia as assessed by the TUNEL assay. The study results support the use of HOST as a tool to identify viable spermatozoa in terms of intact DNA in asthenozoospermia to improve reproductive health outcomes. As sperm swelling grade A has the highest incidence of positive apoptosis staining when compared with other grades, they should not be selected for ICSI, whereas grade D tend to have a lower incidence of positive apoptosis staining and therefore should be prioritized for selection during ICSI.

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