Correlation between Hyaluronan Binding Assay and TUNEL Assay for Sperm DNA Integrity Identification

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ABSTRACT

Objective: To study the correlation between the percentages of HA-unbound sperm and DNA fragmented sperm by TUNEL assay.

Methods: The semen residue from semen analysis was tested by HBA and TUNEL assay.

Results: The mean age of patients included in the study was 34.8 years (± 3.7 years). The proportion of HA-unbound sperm ranged from 11.3% to 24.2%, with a mean of 17.08% (± 3.24%). The range of TUNEL positive in semen samples was 2% to 11.75%, with a mean of 5.78% (± 2.28%). Pearson’s correlation between two tests was 0.848 (p<0.01). Intraobserver variation of the results of HBA ranged from 3.3% to 7.6%, with a mean of 6.23% (± 1.11%). Intraobserver variation of the results of TUNEL assay ranged from 0% to 6.9%, with a mean of 1.54% (± 2.7%). Agreement measuring of each test was determined by using intraclass correlation. The intraclass correlation coefficient of HBA and TUNEL assay were 0.970 (P<0.001) and 0.997 (P<0.001) respectively.

Conclusion: As several studies have found, the binding capacity of sperm to HA is correlated with several sperm parameters. In this study, the strong correlation between the percentages of HA-unbound sperm and TUNEL positive sperm implies, furthermore, that the HA-bound sperm percentage correlates with low levels of DNA fragmentation.

Keywords: Human sperm, hyaluronic acid-binding, DNA fragmentation, TUNEL assay

Siriraj Med J 2008;60:334-338
E-journal: http://www.sirirajmedj.com

Since WHO published “WHO normal values of sperm variables,” a number of articles have been dedicated to the effort to identify optimal parameters with which to evaluate sperm. This effort is necessitated by the fact that a standard semen analysis cannot be applied in all circumstances, especially for couples undergoing assisted reproductive technology (ART). Some of the tests used for sperm evaluation are:

- Kruger strict morphology criteria;
- Aniline blue staining of sperm chromat, histone-protamine replacement test;
- Creatinine kinase and HspA2, biochemical markers for sperm maturity;
- Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), assessing DNA strand breaks (DNA fragmentation) in human sperm.

TUNEL appears to be a useful predictor for a chance of success in ART. It has been shown that sperm DNA fragmentation is inversely correlated with the percentages of oocytes fertilized after IVF. In addition, several studies showed negative effects of DNA fragmentation on reproductive outcomes such as decreasing blastocyst development rates, pregnancy rates, live birth rates, and increasing miscarriage rates. TUNEL thus helps clinicians together with patients to make decisions in difficult situations such as repeated ART failure, and recurrent pregnancy loss. However, the TUNEL assay has disadvantages in that it involves a complex fixing and staining method, it is time consuming–needing at least 1 day, and it requires a fluorescent microscope.

The Hyaluronan binding assay (HBA) was developed for clinical use around 2002. Hyaluronic acid (HA) matrix, a high molecular weight glycosaminoglycan structure, is the major component of the zona pellucida. In HBA, HA is coated onto slides of a commercial HBA. Human sperm attach and remain bound to immobilized HA by HA receptors on their surface.
binding capacity of sperm to HA is correlated with viability, maturity, acrosomal integrity, and is characterized by a low aneuploidy frequency. Huszar et al. demonstrated that only mature sperm with no sperm cytoplasmic retention bind to the zona pellucida.

Although one would assume that mature sperm, as determined by HA binding, should have normal DNA integrity (less DNA fragmentation), the relationship between HBA and TUNEL assay results has not been reported. This study aims to evaluate this correlation.

MATERIALS AND METHODS

This study was a descriptive cross-sectional study performed at the infertility clinic of Siriraj Hospital, Bangkok, Thailand in April 2007. The study protocol was approved by the Ethics Committee of the Faculty of Medicine Siriraj Hospital.

Patients

Semen samples were collected from 19 husbands of infertile couples who came to the infertility clinic, Siriraj Hospital for semen analysis whose results were normal according to WHO normal values of sperm variables.

Semen processing

Patients whose routine semen analysis results qualified them for study participation were counseled, and informed consent was obtained for enrolment. One investigator recorded the results of semen analysis of each sample, number-labeled patients’ semen samples and forwarded these to another, such that the second investigator was blind to the identity of each sample. Samples were diluted with phosphate buffered saline (PBS) to a concentration of 2 x 10^8 sperm/ml, as determined using computer assisted sperm analysis. (TUNEL requires 1-2 million cells per one test only, although samples for HBA can be either fresh or diluted). Two 1 ml samples of each diluted specimen were prepared, one was for HBA by the second investigator and another was sent for TUNEL assay.

Hyaluronan binding assay (HBA)

For HBA, 10 µL of diluted semen were pipetted a nd placed near to the center of the chamber of an HBA kit slide (HYDAK® COATINGS; Biocoat, Inc.), which was covered with a cover slip and incubated 37.6°C for 15 minutes. The incubation time is needed for all of the spermatozoa to contact and bind to the immobilized hyaluronan layer. The cover slip provides a grid of 100, 0.1 x 0.1 mm squares for counting spermatozoa. Spermatozoa in 10 grid squares were evaluated with light microscopy twice to determine the percentage of HA-unbound spermatozoa.

The percentage of HA-unbound spermatozoa is calculated by:

\[
\% \text{ Unbound} = \frac{100 \times \text{Unbound motile}}{\text{Unbound motile + Bound motile}}
\]

TUNEL assay

For TUNEL analysis, the semen sample was subjected to TUNEL staining by using an APO-BrdU™ TUNEL Assay Kit (Molecular Probes, Inc.) by the third investigator. The detail of TUNEL assay is performed as follows:

Cell preparation and fixation

- 0.5 mL of semen sample into 5 mL of 1% (w/v) paraformaldehyde in PBS and place on ice for 15 minutes.
- Centrifuge the sample for 5 minutes at 300 x g and discard the supernatant.
- Wash the sample in 5 mL of PBS then pellet the sample by centrifugation. Repeat.
- Resuspend the sample in 0.5 ml of PBS.
- Add the sample to 5 mL of ice-cold 70% (v/v) ethanol. Let the sample stand for 12-18 hours on ice or in a -20°C freezer.

Detection of apoptotic cells

- Resuspend the sample by swirling the vials. Centrifuge (300 x g) the sample suspensions for 5 minutes and remove the 70% (v/v) ethanol by aspiration carefully.
- Resuspend the sample with 1 mL of Wash Buffer. Centrifuge for 5 minutes at 300 x g and remove the supernatants by aspiration. Repeat.
- Resuspend the sample pellet in 50 µL of the DNA-labeling solution (Mixture of 10 µL of react on buffer, 0.75 µL of TdT enzyme, 8.0 µL of BrdUTP and 31.25 µL of dH2O).
- Incubate the sample in the DNA-labeling solution for 60 minutes at 37°C in a temperature controlled bath. Shake the samples every 15 minutes to keep the samples in suspension.
- At the end of the incubation time add 1.0 mL of Rinse Buffer to the sample and centrifuge at 300 x g for 5 minutes. Remove the supernatants by aspiration.
- Repeat the cell rinsing with 1.0 mL of Rinse Buffer. Centrifuge the sample at 300 x g and remove the supernatants by aspiration.
- Resuspend the cell pellet in 100 µL of the antibody solution (mixture of 5.0 µL of the Alexa Fluor 488 dye-labeled anti-BrdU antibody and 95 µL of Rinse Buffer). Incubate the sample in this solution for 30 minutes at room temperature. Protect the samples from light during the incubation. At the end of the incubation time centrifuge at 300 x g for 5 minutes. Remove the supernatants by aspiration. The cell pellet was deposited onto slide.
- Drop 0.5 mL of the Propidium Iodide/RNase A Staining Buffer to the slide. Incubate the slide for an additional 30 minutes at room temperature. Protect the samples from light during the incubation. Then the slide is ready for evaluation with fluorescent microscopy.

Two hundred spermatozoa on the TUNEL staining slide were evaluated with fluorescent microscopy to determine the percentage of TUNEL-positive spermatozoa. TUNEL-negative spermatozoa fluorescence stained with red color (Fig 1) and TUNEL-positive spermatozoa fluorescence stained brightly in green color (Fig 2) when viewed with filter sets appropriate for fluorescein.

The percentage of TUNEL-positive spermatozoa is calculated by:

\[
\% \text{TUNEL-positive} = \frac{100 \times \text{TUNEL-positive}}{\text{TUNEL-positive + TUNEL-negative}}
\]
Slides were read twice by the same investigator, and the results of each reading were recorded separately. Slides in which the TUNEL readings differed by more than 8 percentage points were excluded from analysis. HBA and TUNEL readings were performed by separate investigators, each blind to the other’s findings.

When all samples were analyzed, semen samples were aligned with the results of semen analysis, HBA and TUNEL assays for data analysis.

Data collection and analysis

The husband’s age, semen analysis results, the percentage of HA-unbound spermatozoa, and the percentage of TUNEL-positive spermatozoa were recorded for each patient.

Statistical analysis was performed by the Computer program “SPSS for Microsoft Windows version 10.0”. The percentages of HA-unbound and TUNEL-positive spermatozoa were plotted in the scatter diagram and analyzed by Pearson’s correlation. The results of semen analysis are presented as mean and standard deviation (mean ± SD.).

RESULTS

A total of 19 semen samples were evaluated. The mean age of patients included in the study was 34.8 years (± 3.7 years). The values of basic semen variables were shown in Table 1. The range of TUNEL positive in semen samples was 2% to 11.75%, with a mean of 5.78% (± 2.28%). The proportion of HA-unbound sperm ranged from 11.3% to 24.2%, with a mean of 17.08% (± 3.24%).

The percentages of TUNEL positive and HA-unbound sperm were plotted in a scatter diagram as presented in Figure 3. The scatter diagram showed a trend of linear association between the two variables. The normal parameters test of two variables was done and both showed a normal distribution. Pearson’s correlation between two tests was 0.848 (p<0.01).

Intraobserver variation of the results of HBA ranged from 3.3% to 7.6%, with a mean of 6.23% (± 1.11%). Intraobserver variation of the results of TUNEL assay ranged from 0% to 6.9%, with a mean of 1.54% (± 2.7%). No semen sample was excluded from the study by intraobserver variation of TUNEL assay that was more than 8.

Agreement measuring of each test was determined by using intraclass correlation. An intraclass correlation coefficient of HBA and a TUNEL assay were 0.970 (P<0.001) and 0.997 (P<0.001) were determined respectively.

DISCUSSION

Despite the extent of ART development to date, there is still no adequate single test to assess sperm quality, male fertility or predict the chance of success when employing ART. Clinicians sometimes combine a variety of sperm evaluation tests to complement standard semen analysis. Many tests have been developed to evaluate sperm DNA damage or DNA fragmentation, and the degree to which these parameters predict male fertility has been extensively studied.

The TUNEL assay was originally designed for measuring DNA fragmentation occurring during apoptosis, and was then applied for detecting DNA strand breaks in human sperm. Recent studies used the TUNEL
TABLE 1. Semen variables.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>Volume (ml)</td>
<td>1.90</td>
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<tr>
<td>Sperm concentration (x10³/ml)</td>
<td>40.47</td>
</tr>
<tr>
<td>Total sperm count (x10⁹)</td>
<td>72.56</td>
</tr>
<tr>
<td>Vitality (%)</td>
<td>83.11</td>
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<tr>
<td>Morphology (%)</td>
<td></td>
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<tr>
<td>Normal morphology</td>
<td>38.53</td>
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<tr>
<td>Head defect</td>
<td>48.05</td>
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<tr>
<td>Mid piece defect</td>
<td>8.47</td>
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<tr>
<td>Tail defect</td>
<td>4.32</td>
</tr>
<tr>
<td>Cytoplasmic droplet</td>
<td>0.79</td>
</tr>
<tr>
<td>Motility (%)</td>
<td></td>
</tr>
<tr>
<td>Category A</td>
<td>43.47</td>
</tr>
<tr>
<td>Category B</td>
<td>15.68</td>
</tr>
<tr>
<td>Category C</td>
<td>20.95</td>
</tr>
<tr>
<td>Category D</td>
<td>19.68</td>
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</tbody>
</table>

The present study shows a statistical correlation between the percentages of HA-unbound sperm and TUNEL positive sperm. This finding implies that the HA-bound sperm parameter correlates with low levels of DNA fragmentation. Several studies have found that binding capacity of sperm to HA is correlated with several sperm parameters. This finding supports the use of HBA for a general assessment of male fertility, or in special situations like assessment of the male factor in recurrent pregnancy loss couples. HBA is suitable for this purpose because it reflects multiple sperm parameters concomitantly, is easy to use, and requires only light microscopy.

As TUNEL results have shown correlation with blastocyst development and pregnancy rates, so HBA should be further studied as a predictor of these events, which would inform the likelihood of success in more elaborate undertakings like blastocyst culture. Additionally, HBA may have a role in predicting ART success, and so could be a useful counseling tool for couples considering this intervention.

Another possible application of HBA is its use for sperm selection for intracytoplasmic sperm injection (ICSI). ICSI uses normal sperm morphology for sperm selection for injection. A previous study shows that HBA and sperm morphology statistically are significantly related to fertilization rates. However, this study demonstrated that HBA was less significant than normal sperm morphology for predicting the fertilization rate. In ICSI, selection of a normal spermatozoon can be accomplished by observing its shape, light refraction and motion pattern, but this evaluation is difficult and highly subjective. It has been suggested that ICSI might cause an increase in malformation, adverse development effects and poorer pregnancy outcomes, which may be attributable to poor sperm selection. For this reason, sperm selection by HBA, which would suggest high quality sperm by multiple parameters—especially chromosomal statuses and DNA integrity, which cannot be determined by simple morphological assessment—should help to improve ICSI outcomes. One trial has been conducted using HA-bound sperm for ICSI in pigs, and it showed significantly lower chromosome abnormality rates in pig embryos. Further studies along these lines are warranted to assess the suitability of this approach in humans.

CONCLUSION

The present study shows a statistical correlation between the percentages of HA-unbound sperm and TUNEL positive sperm. This finding implies that the HA-bound sperm parameter correlates with low levels of DNA fragmentation. As several studies have found, the binding capacity of sperm to HA correlates with several sperm parameters. All of these findings support the use of HBA for assessment of sperm DNA and also the further clinical trial of the application of HBA in ART.

REFERENCES


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