

HBA as a Potential Alternative to the TUNEL Assay for Assessment of DNA Integrity in Asthenozoospermic Spermatozoa

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ABSTRACT

Objective: This study was conducted to evaluate the appropriateness of the hyaluronan binding assay (HBA) for assessment of DNA integrity indirectly in asthenozoospermic spermatozoa, by studying its correlation with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.

Methods: Thirty asthenozoospermic semen samples of infertile couples were recruited. Semen analysis was performed before being processed by HBA and the TUNEL assay. All the semen parameters were recorded and analyzed.

Results: The mean age of the patients was 38.1 ± 6.4 years. The average total and progressive motility was $33.5 \pm 11.0\%$ and $21.9 \pm 6.6\%$, respectively. The mean TUNEL-positive and hyaluronan-bound sperm were $17.8 \pm 6.4\%$ and $57.2 \pm 14.5\%$, respectively. Pearson correlation coefficient showed a high negative correlation between HBA and the TUNEL assay ($r = -0.73$, $p < 0.001$). The intraclass correlation coefficients revealed a high level of agreement between two observers who evaluated the TUNEL-positive and hyaluronan-bound sperm [0.81 (95% CI: 0.65–0.91) and 0.92 (95% CI: 0.84–0.96)], respectively.

Conclusion: The study results showed a high negative correlation between HBA and the TUNEL assay. This suggests that HBA may be used as a viable intact DNA spermatozoa identification method, as a potential alternative to the TUNEL assay, in asthenozoospermia sperm.

Keywords: Spermatozoa; asthenozoospermia; hyaluronan binding assay; TUNEL assay; infertility and DNA fragmentation (Siriraj Med J 2018;70: 70-76)

INTRODUCTION

Asthenozoospermia is one of the causes of male infertility. In a fresh ejaculate, the lower limits of normal semen analysis are defined as 32% progressive motility (5th centile, 95%CI 31-34) and 40% total motility (5th centile, 95%CI 38-42).¹ The prevalence of asthenozoospermia has been reported to be as high as 18.71–24.19%.^{2,3} This could be associated with certain factors, such as advanced age,⁴

varicocele,⁵ smoking, alcohol consumption, drugs, toxic substances and chemotherapy. Sperm velocity has been suggested to be a promising indicator of sperm function and fertility.⁶ Diminished sperm motility prevents the sperm from transiting itself through the epididymis, vagina and fallopian tubes to the oocyte, to penetrate the zona pellucida for fertilization.² To overcome the low fertilization rate, intracytoplasmic sperm injection

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(ICSI) has been adopted to select the morphologically normal, motile sperm in asthenozoospermic males. However, a real-time PCR study has shown higher DNA fragmentation in asthenozoospermia than in normal spermia.⁷ Several studies have also suggested the association of sperm motility with DNA fragmentation.^{8,9} Because ICSI bypasses the natural selection barrier for fertilization, the possibility of transmitting damaged sperm genetic material to conceptus increases, which may affect fetal or postnatal life. Several studies have reported that sperm DNA damage is associated with decreased fertilization rate, embryo quality, implantation rate, increased miscarriage rate, childhood diseases and cancer.¹⁰⁻¹⁶

DNA integrity analysis is a good diagnostic test for sperm quality, and is useful for predicting pregnancy outcomes.^{17,18} High level of DNA fragmented sperm is associated with a significant increase in miscarriage rate¹⁹ and a lower live birth rate.²⁰ Therefore, finding methods for discriminating DNA-intact spermatozoa from the asthenozoospermic samples, to improve the chance of conception and reproductive health outcomes is a challenging topic.

One of the commonly used standard techniques to reveal sperm DNA integrity is the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay.¹² However, in addition to being time-consuming, requiring special skills and expensive, it is toxic to spermatozoa. Therefore, it is not a suitable test for spermatozoa selection for ICSI.

Accordingly, the appropriate method is to use non-destructive DNA tests or tests that can predict DNA fragmentation indirectly, for example, the hyaluronan binding assay (HBA), which has some advantages. First, it is a test for choosing viable mature sperm in a fresh semen sample. Hyaluronan is the major component of the cumulus oophorus matrix and zona pellucida surrounding the oocyte. Sperm binds to hyaluronan only after it completes its plasma membrane remodeling, cytoplasmic extrusion and nuclear maturation, because hyaluronic acid binding sites fail to appear upon incomplete spermiogenesis.¹⁶ The plasma membrane remodeling ability of mature sperm is necessary for facilitating the zona pellucida or hyaluronic acid binding site.²¹ This binding process is important for the selection of the functionally most competent sperm for fertilization in vivo.¹⁶ Biochemical markers indicating sperm development and function, such as sperm creatinine phosphokinase activity and heat shock protein family A (Hsp70) member 2 (HspA2), are properly expressed in hyaluronan-bound sperm. In vitro, HBA is used to evaluate the sperm maturity through assessment of the binding capacity of its head to hyaluronan. Second,

HBA is a simple, quick test that provides a cost-effective benefit. Third, HBA has been reported to be associated with the fertilization rate and pregnancy outcomes.²²⁻²⁵ As the hyaluronan binding ability is related to many semen parameters, such as viability, acrosomal integrity and low aneuploidy frequency,^{26,27} this test could be useful for predicting the ability of spermatozoa to fertilize oocytes in vivo, as well as ICSI. Despite there are publications demonstrating poor correlation between HBA test and sperm penetration assay (SPA),²⁸ which assesses direct fertilising capacity of sperm such as capacitation, acrosome reaction, fusion and penetration of the oolemma and chromatin decondensation; it is controversial whether HBA test would be a sufficient indirect analysis to examine fertilising ability in normal semen samples instead of SPA.²⁹ Finally, DNA fragmentation is associated with HBA. A recent study has revealed a correlation between HBA and the TUNEL assay²⁷ in normal semen. Several studies have reported an association of DNA fragmentation with HBA.^{22,30} Higher clinical pregnancy rate and birth rate were reported as well.³¹ However, there has been insufficient data regarding this association in asthenozoospermia.

The TUNEL assay and HBA work on different definitions of good sperm. The former is used to find intact genetic material, while the latter is used to find mature sperm. This study was conducted to reveal whether the mature sperm is associated with intact DNA contents inside. The purpose of our research was to clarify the correlation between HBA and the TUNEL assay in asthenozoospermia. The results of this study will help in the decision of using HBA to assess sperm DNA integrity, and thus, facilitate the routine sperm selection for ICSI and improve reproductive health outcomes.

MATERIALS AND METHODS

This cross-sectional study was conducted at the Infertility Unit, Department of Obstetrics and Gynecology, Siriraj Hospital from March 2015 to August 2016. The study protocol was approved by the hospital institutional review board (Si 128/2015). The sample size was based on the results of a previous study, which reported a correlation of 0.848 between HBA and the TUNEL assay for DNA integrity.²⁷ The sample size was calculated to achieve a 95% confidence level and 80% power.

After a written informed consent was granted, the male partners of infertile couples were asked to collect a semen sample by masturbation. Semen was left for liquefaction at room temperature for 30 min. Semen analysis was then performed by computer-aided sperm analysis (CASA; Hamilton Thorne Inc., Beverly, MA, USA). Samples with asthenozoospermia, diagnosed according

to the World Health Organization 2010 criteria,¹ were included in this study. Baseline information and semen analysis results were collected.

Semen processing

Two aliquots were extracted from each liquefied semen sample and subjected to HBA and the TUNEL assay within 90 min of semen collection. Interpretation was made twice on each sample by well-trained personnel without knowledge of the results of each assay, based on 200 spermatozoa.

HBA

HBA testing was performed following the instructions of the manufacturer. Seven to ten microlitres of liquefied semen sample were pipetted and placed near the center of the chamber of an HBA kit slide (HBA[®] Sperm-Hyaluronan Binding Assay; Biocoat, Inc., Horsham, PA, USA), which was covered with a Cell-Vu gridded cover slip and incubated at 20–30°C for at least 10 and not more than 20 min. The incubation time is needed for all the spermatozoa to contact and bind to the immobilized hyaluronan layer. The Cell-Vu gridded cover slip provides a grid of 100 0.1×0.1 mm² squares for counting spermatozoa. Spermatozoa in 10 grid squares were evaluated with light microscopy twice, to determine the percentage of hyaluronan-bound spermatozoa.

The percentage of hyaluronan-bound spermatozoa was calculated by the following formula:

$$\% \text{ bound} = \frac{\text{Bound motile sperm}}{\text{Bound motile sperm} + \text{Unbound motile sperm}} \times 100$$

TUNEL assay

For TUNEL analysis, the semen sample was subjected to TUNEL staining using an In Situ Cell Death Detection Kit (Fluorescein; Roche, Germany). The TUNEL assay was performed following the instructions of the manufacturer: Semen samples were put on slides. The air-dried samples were fixed with a freshly prepared Fixation Solution [4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4] for an hour at 15–25°C. The slides were then rinsed with PBS and incubated in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice (2–8°C). Then, the slides were washed twice with PBS and dried around the sample. Fifty microliters of TUNEL reaction mixture were placed on the samples. Then 10 µL of 4',6-Diamidino-2'-phenylindole dihydrochloride was added to the samples as well. To ensure a homogeneous spread of the TUNEL reaction mixture across the cell monolayer and to avoid evaporation, samples were

covered with parafilm or a coverslip during incubation. The slides were incubated in a humidified atmosphere for 60 min at 37°C in the dark. Next, the slides were rinsed three times with PBS. Samples were directly analyzed by flow fluorescence microscopy.

Two hundred spermatozoa on the TUNEL staining slide were evaluated with fluorescence microscopy (Olympus fluorescence microscope Bx51, Melville, NY, USA) at 40x magnification, to determine the percentage of TUNEL-positive spermatozoa. TUNEL-negative spermatozoa were stained blue and TUNEL-positive spermatozoa were stained brightly green (Fig 1), which indicated DNA fragmentation.

The percentage of TUNEL-positive spermatozoa was calculated by the formula:

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

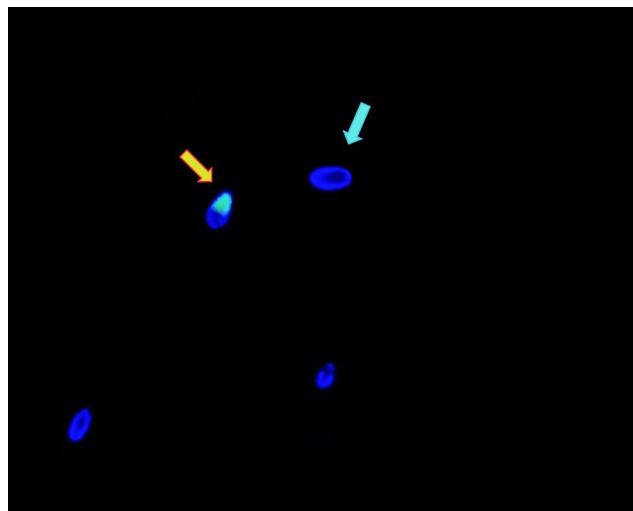


Fig 1. TUNEL-negative spermatozoa was stained by DAPI, the blue fluorescence (light blue arrow), while TUNEL-positive spermatozoa was stained brightly in green fluorescence (yellow arrow). In this picture, the sperm was partially stained by TUNEL indicating partial damage of sperm DNA.

Statistical analysis

The characteristics of the patients and their semen are presented as mean ± SD, or median with interquartile range, as appropriate. Intraclass correlation coefficient (ICC) was used to evaluate the reliability of the TUNEL and HBA scores. Pearson correlation coefficient was used to assess the correlation between the HBA and TUNEL scores. The quantitative variables between the HBA groups were compared using the unpaired t-test or Mann-Whitney U test, as appropriate. Data were analyzed using PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

RESULTS

In total, 463 infertile males were screened for eligibility, of which 30 were enrolled in this study. The mean age of the patients was 38.1 ± 6.4 years. The basic semen parameters are displayed in [Table 1](#). The mean semen volume was 3.0 ± 1.4 mL. The median sperm concentration was $15.5 \times 10^6/\text{mL}$. The mean total and progressive motility were $33.5 \pm 11.0\%$ and $21.9 \pm 6.6\%$, respectively. The mean normal sperm morphology was

$8.9 \pm 5.9\%$. The mean TUNEL-positive and hyaluronan-bound sperm in the semen samples was $17.8 \pm 6.4\%$ and $57.2 \pm 14.5\%$, respectively. In the previous report that studied in the normal semen, there were $5.78 \pm 2.28\%$ of TUNEL-positive sperms and $17.08 \pm 3.24\%$ of HBA-unbound sperms.²⁷ The results in the asthenozoospermia seemed to be higher than the normal semen. This suggested the higher DNA fragmentation and lower matured sperms in asthenozoospermia.

TABLE 1. Characteristics of semen parameters (n = 30). Data are presented as mean \pm SD or median (min, max), as appropriate.

Factor	Mean \pm SD/Median (IQR)
pH	7.8 ± 0.3
Volume (mL)	3.0 ± 1.4
Sperm concentration ($10^6/\text{mL}$)	15.5 (1.5, 67)
Total sperm count (10^6)	37.1 (3.2, 250.5)
Normal morphology (%)	8.9 ± 5.9
Vitality (%)	49.3 ± 12.5
Motility (%)	
Total	33.5 ± 11.0
Progressive	21.9 ± 6.6
Non-progressive	11.6 ± 8.0
Immotile sperm (%)	66.8 ± 11.4

There was no correlation found between the proportion of the TUNEL-positive sperm and the sperm motility ($r = 0.2$, $p = 0.288$). However, the percentage of the HBA-bound sperm and the sperm motility ($r = -0.317$, $p = 0.08$) tended to be correlated ([Fig 2](#)). The percentages of TUNEL-positive and hyaluronan-bound sperm were plotted in a scatter diagram ([Fig 3](#)). Pearson correlation coefficient showed a strong negative correlation between the HBA and the TUNEL assay results ($r = -0.73$, $p < 0.001$). The intraclass correlation coefficient revealed that there was an adequate level of agreement between two observers who evaluated the percentages of TUNEL-positive and hyaluronan-bound sperm [0.81 (95% CI: 0.65–0.91) and 0.92 (95% CI: 0.84–0.96)], respectively.

DISCUSSION

Since the first IVF baby was born in 1978, the assisted reproduction technology has been developed continuously. Many methods for semen preparation have

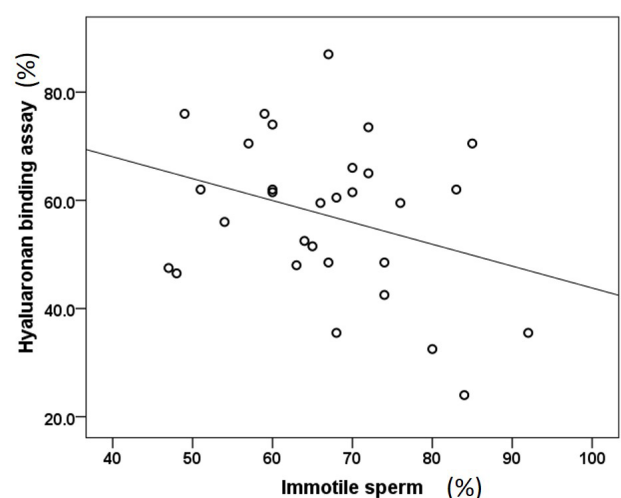


Fig 2. Scatter plots of the percentages of HBA-bound sperm and the sperm motility.

The percentages of HBA-bound sperm tended to be negatively correlated with the immotile sperm number ($r = -0.317$, $p = 0.08$), however, is not statistically significant.

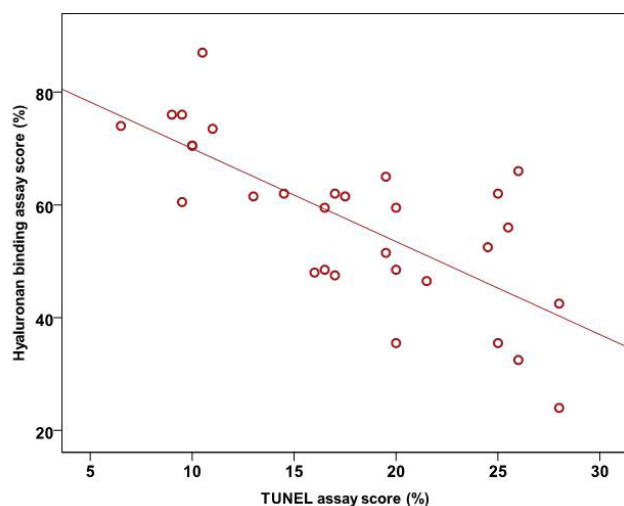


Fig 3. Scatter plot of the percentages of TUNEL-positive and hyaluronan-bound sperm.

The percentages of TUNEL-positive and hyaluronan-bound sperm were plotted in a scatter diagram. Pearson correlation coefficient showed a high negative correlation between the HBA and the TUNEL assay results ($r = -0.73$, $p < 0.001$). The intraclass correlation coefficient revealed that there was a high level of agreement between two observers who evaluated the percentages of TUNEL-positive and hyaluronan-bound sperm [0.81 (95% CI: 0.65–0.91) and 0.92 (95% CI: 0.84–0.96)], respectively.

been developed to select the best viable spermatozoa for fertilization. Injection of damaged sperm was minimized by conventional semen preparation. Both density gradient centrifugation and swim up techniques help filter the sperms with less DNA damage.³² Combination of a variety of sperm evaluation tests may provide a better selection over standard semen preparation. Selection based on the morphology and motility is used worldwide. However, several studies have reported that spermatozoa with normal morphology may have DNA fragmentation.^{33,34} In the case of severe asthenozoospermia, it is difficult to distinguish between live and dead sperm. The mechanical touch technique, which assesses viability by observing tail flexibility and tail recovery after touching it with an ICSI pipette, provides a fertilization rate of 30.3%–73.4%.^{35,36} However, there are variations in the criteria for identifying viable spermatozoa using this technique. Thus, a technique for choosing viable sperm with intact DNA in asthenozoospermia is required.

The association between the mature sperm and the integrity of the genetic material was studied. The results showed a high negative correlation between the HBA and the TUNEL assay results [$r(28) = -0.726$, $p < 0.001$]. This is comparable to the results reported by previous studies, which were conducted on normal semen and generally infertile males.^{22,27} The difference in

the strength of the correlation compared with the study conducted on normal semen is probably because of the difference in the study population, as the present study only enrolled asthenozoospermia patients.

Asthenozoospermia contains a high number of immotile sperm, which includes both the live immotile and the apoptotic or dead sperm. These apoptotic nonviable germ cells were suspected to have DNA fragmentation. However, this study did not find the association between the TUNEL-positive sperm and sperm motility. The result suggested that DNA damage is caused similarly in both motile and immotile groups. On the contrary, the correlation between the HBA and TUNEL test suggested the possibility that DNA damage is more frequent in the immature motile sperm than mature motile sperm in the asthenozoospermia patients. This is in accordance with other studies that have reported low DNA fragmentation in hyaluronan-bound sperm,^{24,30,37,38} using green acridine orange fluorescence, Halosperm kit and the TUNEL assay. Several studies reported that both total and progressive sperm motility and normal morphology were highly correlated with HBA scores.^{39–41} In the present study, we found that the percentages of HBA-bound sperm had a trend of negative correlation with the immotile sperm number, although it was not statistically significant (Fig 2). This may due to the small sample size for the secondary outcome assessment. Sperm maturation and DNA fragmentation have been suggested as being closely related. Experiments have shown that diminished maturity sperm possess defects in both histone-protamine replacement and DNA fragmentation.⁴² The maturation process of spermatogenesis is related to many substances, especially the HSP70 family of proteins (HspA2 in men). HspA2 is highly expressed during two major stages, first in spermatocytes as a meiotic component within the synaptonemal complex, second in elongating spermatids during terminal spermiogenesis.²¹ Low HspA2 expression during spermiogenesis causes a synaptonemal complex defect, which leads to chromosome aneuploidy. Inadequate delivery of DNA repair enzymes, which may be due to the diminished HspA2 chaperone activity, leads to DNA fragmentation.²⁵ Moreover, immature sperm containing excessive cytoplasm, but low HspA2 level, express insufficient zona binding sites and are unable to bind to hyaluronan.⁴³ Therefore, live sperm that bind to hyaluronan reflect their maturity and are indirectly suggested to possess intact genetic material, and lack persistent histones and apoptosis.

Thus, HBA, is a simple, time-saving, non-toxic and cost-effective method, which is reasonable to be adopted for evaluating DNA fragmentation indirectly instead

of the TUNEL assay in asthenozoospermia patients. It may help in sperm selection for ICSI, and consequently facilitate our routine fertility treatment and improve reproductive health outcomes.

There were some limitations in this study. The study was conducted to examine the correlation between two tests. As there are several studies which reported lower DNA fragmentation in HBA bound sperm, the DNA fragmentation proportion in the total sperm population and the hyaluronan-bound group in this study were not assessed. Despite the suggestion of intact DNA using HBA selection, its clinical value for fertility and pregnancy outcomes needs to be further evaluated.

In conclusion, the study results showed a strong negative correlation between HBA and the TUNEL assay. The results support the idea that HBA may be used as a viable intact DNA spermatozoa identification method in asthenozoospermic patients.

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