Additional Hyaluronic Acid Binding Selection Decreased Sperm DNA Damage after Conventional Semen Preparation in Infertile Patient with Abnormal Semen Analysis

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ABSTRACT

Objective: To study the benefit of combining conventional sperm preparation methods with hyaluronic acid binding in selecting spermatozoa with less DNA damage in infertile patients with abnormal semen analysis.

Methods: Forty-six semen samples obtained from infertile men, containing two or more abnormalities regarding concentration, motility or morphology, were enrolled in the study. Baseline semen analysis was performed and recorded for all samples. Each sample was divided into 3 aliquots, i.e. the control group / fresh sample before preparation (Group 1), the conventional sperm preparation group including both density gradient centrifugation and swim-up (Group 2), and the conventional method combined with hyaluronic acid selection group (Group 3). The DNA integrity of sperm obtained by each different test group was assessed using TUNEL assay.

Results: Less sperm DNA damage was observed in Group 2 compared to Group 1, whereas Group 3 displayed significantly lower sperm DNA damage compared to Group 2 (9.4% vs. 27.0%, $p < 0.001$).

Conclusion: Sperm DNA damage was significantly reduced after the inclusion of hyaluronic acid binding in conventional sperm preparation method in abnormal semen samples.

Keywords: DNA damage; sperm preparation; density gradient centrifugation; swim-up; hyaluronic acid binding; TUNEL (Siriraj Med J 2018;70: 213-218)

INTRODUCTION

Infertility has developed into a public health issue affecting around 6.8-38.6% of reproductive couples worldwide, depending on different diagnostic criteria.[1] Male factor alone is estimated to be responsible for 20-30% of these cases.[2] Sperm quality is commonly described by the parameters of concentration, motility and morphology. Deviation of these parameters from the reference thresholds is correlated with poor quality and usually fertility problems.[3] Poor sperm quality (varying degrees of oligo-astheno-teratozoospermia) is frequently associated with increased sperm DNA fragmentation in infertile males.[4,5]

Many factors can influence semen quality, but the primary mechanism inducing DNA fragmentation is the post-testicular damage by free oxygen species, which usually occurs during sperm transportation through the epididymis.[6,7] However, normal semen samples obtained

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from infertile males, also display higher DNA fragmentation rate compared with fertile controls.[7,8] Sperm DNA damage may have several effects including fertilization failure and poor embryo development. Additionally, data supports the association of sperm DNA fragmentation with low live-birth rates,[9,10] increased pregnancy loss after IVF or intracytoplasmic sperm injection (ICSI),[11] poor embryo quality and lower implantation rate.[12-14]

ICSI is an ART tool designed to overcome fertilization failure of poor quality sperm. Sperm selection during ICSI usually depends on sperm motility and morphology. Nevertheless, motile spermatozoa with normal morphology may also carry fragmented DNA. This phenomenon is even more popular in poor quality semen samples.[4]

Several tests have been designed to assess DNA integrity, although none is generally accepted as the best diagnostic tool to predict or optimize clinical management.[15] Terminal deoxynucelotidyl transferase–dUTP nick end-labelling (TUNEL) assay is one of these methods providing great accuracy for quantifying apoptotic germ cells.[16,17] However, TUNEL is not suitable for DNA integrity assessment of viable sperm during ICSI, since the testing solutions used are toxic to the sperm.

Hyaluronic acid binding assay (HBA) is developed for selection of mature spermatozoa imitating the natural process of mature sperm binding on the cumulus oophorus complex of the oocyte which is composed of polymerized hyaluronic acid and glycoproteins.[18] Hyaluronic acid bound spermatozoa have been reported to carry low DNA fragmentation, less chromosomal aneuploidies and better nucleus morphology.[19,20] Hence, HBA method may be able to, indirectly, predict sperm DNA integrity while at the same time allowing the selected sperm to be used for ICSI thereafter. No adverse effects have been reported on either fertilization or embryo development following the use of hyaluronic acid bound spermatozoa in clinical IVF settings.[21]

The aim of this study was to compare the presence of DNA damage in spermatozoa retrieved by two different preparations methods prior to ICSI process, namely the conventional method (density gradient centrifugation followed by swim-up) and the conventional method combined with HBA method in infertile patients with abnormal semen analysis. The DNA fragmentation was evaluated by TUNEL assay.

MATERIALS AND METHODS

Study Design and Patient Selection

This was an experimental study, conducted at the infertility unit of a university hospital. The study protocol was approved by hospital institutional review board (Si 591/2012). The inclusion criteria contained healthy men, over 18 years of age, non- smokers, without medical history of receiving radiotherapy or chemotherapy and with abnormal semen analysis. Samples with two or more abnormal semen parameters (concentration, motility and morphology) according to World Health Organization (2010) criteria[3] were included. The subjects who had leukocytospermia or sperm motility < 5% were excluded from this study.

After baseline semen analysis was completed, each semen sample was divided into 3 aliquots. Group 1 used fresh smear as control. In Group 2, semen was prepared by the conventional method where density gradient centrifugation was followed by swim-up technique. In Group 3, semen prepared by the conventional method underwent HBA testing as well. Each group of semen sample was assessed for DNA damage by TUNEL assay.

Density gradient centrifugation and swim up

Density gradient conical tubes were prepared by adding 2 ml of 80% and 40% of percoll buffer (Percoll™ PLUS, GE Heathcare, Uppsala, Sweden) in lower and upper layers, respectively. The liquefied semen was layered onto the gradient. All samples were centrifuged at 300 x g for 20 minutes. Then the supernatant was gently removed, leaving the pellet at the bottom. A new pipette was used to aspirate the pellet and place it in 1ml of sperm media. For the swim up process 0.5 ml of Earle's balanced salt solution (Gibco™, Paisley, Scotland, UK) was placed over the sperm layer and was incubated in 37°C for 30 minutes. The sperm in the upper 0.5 ml was obtained for analysis or further treatment.

Hyaluronic acid binding method

The HA binding method was performed by placing 10 µL of SpermSlow™ media (Origio Medicult Media, Denmark) at the bottom of a pre-warmed at 37°C, ICSI dish. Then the prepared semen sample was placed close to the media. A junction between the sperm droplet and the SpermSlow™ was created by the tip of pipette. The ICSI dish was covered immediately with pre-equilibrated liquid paraffin oil and subsequently placed in a 5% CO_2, 37°C incubator for 15 minutes. The injection pipette was washed by pre-equilibrated sperm preparation medium before being used to select the spermatozoa. The motile sperm bound on HA were picked. Around 500 spermatozoa were collected and fixed for DNA assessment.

DNA damage analysis with the TUNEL assay

The DNA fragmentation was evaluated using the TUNEL assay (In Situ Cell Death Detection Kit, Fluorescein,

Roche, Mannheim, Germany). The slides were initially fixed in 4% paraformaldehyde in Phosphate Buffer Saline solution for 1 hour. Then all slides were rinsed with phosphate buffer saline (PBS) and incubated in 0.1% triton- X 100 in 0.1% sodium citrate for 2 min on ice (2-8°C). Afterwards, slides were rinsed twice with PBS and incubated in 50 µL of TUNEL mixture in a humidified atmosphere, at 37°C, in the dark for 60 minutes before being washed three times with PBS. After being dried at room temperature, counterstain was performed with DAPI II solution (Abbott™, Ilinois, USA) which was then evaluated under fluorescence microscope (Imager-Z1; Zeiss, Göttingen, Germany). At least 500 spermatozoa were assessed per sample in random fields. TUNEL-positive spermatozoa exhibited a strong nuclear green fluorescence. DNA damage degree was expressed as a percentage of TUNEL-positive spermatozoa. The slides were assessed by two technicians and the mean of the results was used for calculation.

Statistical analysis

The degree of DNA damage between the two methods was assessed by Chi-square test. The correlation between semen parameters and DNA damage were analyzed by Pearson's correlation coefficient or Spearman's rank correlation in the data without normal distribution. The data analysis was performed using Statistical Programs for Social Sciences (SPSS Inc. version 15.0, Chicago, IL). A P value of < 0.05 was considered statistically significant. Sample size was calculated based on the data of a previous study,[20] which reported 11% DNA damage in swim-up technique and 5.3% in the HBA group. At type I error of 0.05 and allowable error of 0.1, at least 46 samples were needed for this study.

RESULTS

Totally 127 participants were eligible for the study. Eighty-one participants were excluded due to containing only one abnormal semen parameter. Therefore, forty-six participants were enrolled in the study. There were 34 semen samples with two abnormal semen parameters and 12 samples contained all three abnormalities on the aspect of concentration, motility and morphology. All subjects included in this study were recruited after providing informed consent.

The basic characteristics of all subjects and the semen parameters of the samples are summarized in Table 1. The mean age of participants was 34.9 ± 6.0 years. The mean abstinence time was 4.1± 2.2 days. The mean semen volume, concentration, motility and normal morphology was 2.1 ± 1.4 mL, 14.7 ± 18.8 x 10^6/mL, 26.0 ± 11.4% and 5.0 ± 3.6% respectively.

The DNA damage in fresh semen (Group 1) was measured at 39.0% by TUNEL assay. The sperm DNA damage in Group 2 (27%) was significantly lower than Group 1 ($P < 0.001$) and was markedly reduced in Group 3 (9.4%) compared to Group 2 ($P < 0.001$), as well. The fresh sperm DNA damage was similar in participants who had two (Group A) and three (Group B) abnormal semen parameters (38.9% and 39.3%, respectively). The conventional preparation significantly reduced DNA fragmentation in both Groups A and B to 27.1% and 26.8%, respectively. The addition of HBA reduced DNA damage in Group A and B even further to the lowest rate found in this study, i.e. 10.1% in Group 2 and 7.5% in Group 3 ($P < 0.001$) (Fig 1).

From univariate analysis for factors of sperm DNA damage, there was no linear relationship between semen parameters and DNA damage (Table 2).

TABLE 1. Basic characteristics of recruited subjects

	Mean ± SD	Median	Range
Age (years)	34.9 ± 6.0	35	24 – 50
Abstinent time (days)	4.1 ± 2.2	4	2 – 7
Semen volume (mL)	2.1 ± 1.4	1.60	1 – 7
Sperm concentration (million/mL)	14.6 ± 18.8	8	0.1 – 73
Sperm motility (%)	26.0 ± 11.4	27	3 – 50
Normal sperm morphology (%)	5.0 ± 3.6	4	0 – 16
WBC in semen (million/mL)	0.3 ± 0.2	0.2	0 - 0.8

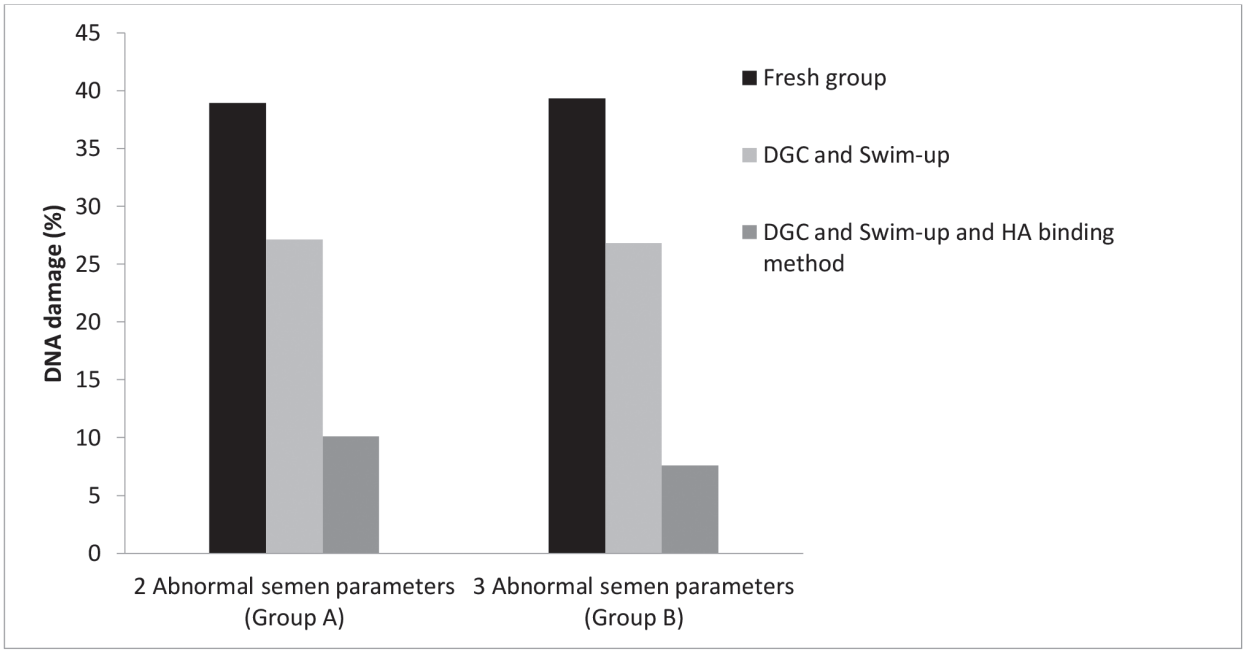


Fig 1. Comparison of DNA damage between conventional method of sperm preparation and additional HBA method in samples with 2 and 3 abnormal semen parameters by TUNEL assay. The DNA fragmentation level in semen containing 2 or 3 abnormalities were similar. After preparation with conventional (DGC and swim up) and additional HBA method, the DNA damage rate was significantly decreased in both groups.

TABLE 2. Univariate analysis for factors of sperm DNA damage using Spearman's rank correlation

Factors	Correlation coefficient (r values)	P value
Age (years)	-0.09	P = 0.51
Abstinent time (days)	-0.01	P = 0.93
Semen volume (mL)	-0.51	P = 0.73
Sperm concentration (million/mL)*	-0.06	P = 0.68
Sperm motility (%)	0.12	P = 0.42
Normal sperm morphology (%)	0.01	P = 0.96
WBC in semen (million/mL)	0.05	P = 0.73

DISCUSSION

Sperm DNA damage influences a series of fertility processes, from fertilization to delivering a healthy baby. Sperm fragmentation may have a negative impact on clinical pregnancy after both IVF and ICSI.[22] Therefore, choosing normal and free of DNA damage sperm for fertilization is a crucial step in creating a new life through assisted reproduction technology. Although sperm motility and morphology are associated with fertilization rate[23, 24] and pregnancy outcome,[25] they are not always representative of the sperm DNA integrity. This study suggests that an additional HBA step following density gradient centrifugation and swim up may help in selecting spermatozoa with lower DNA damage.

Fresh sperm analysis showed higher DNA damage in our study (39%) compared to previous ones, which reported only 6-23%.[4] This may be due to differences in our sample and the reference values used for semen analysis.[4] This study used the WHO 2010[3] cut-off values while the previous study used the WHO 1999 criteria.

Therefore, our study showed more severe abnormalities in semen parameters as lower mean of total motility and normal morphology were applied.

This study also demonstrated the degree of sperm DNA damage post different preparation methods in infertile patients with abnormal semen analysis. Both experimental groups (Group 2 and Group 3) showed reduced sperm DNA fragmentation. To begin with, Group 2 displayed a DNA damage degree of 27% compared to 39% of fresh sample. These results are a consequence of the preparation method itself. To be more specific, most healthy sperm were initially centrifuged to the precipitate and then the most motile sperm swam up to the upper layer of the medium during swim-up process. Thus, most apoptotic sperm were eliminated after conventional preparation. Sperm DNA fragmentation was significantly decreased in Group 3 (9.4%) compared to Group 2 (27%), representing the benefit of an additional HBA step in improving sperm quality. The significant reduction in DNA fragmentation observed in our study after the addition of HBA is consistent with previous studies.[20, 26, 27] DNA damage rates may vary between this study and the former ones, but differences probably originate from the different patient inclusion criteria and different protocols of measuring DNA fragmentation (TUNEL, sperm chromatin dispersion (SCD) test, Chromomycin A3 (CMA3) staining, etc.). For example, Parmegiani[20] studied normozoospermic infertile patients and used the SCD test while we recruited subjects with abnormal semen analysis and used the TUNEL assay.

Regarding sperm DNA damage assessment, there is no consensus on the particular test that offers the most accurate diagnosis to optimize clinical management.[15] The available direct DNA evaluation tests are toxic to sperm and therefore not useful during ICSI. Previous study of our university reported an association of HBA testing with TUNEL assay, and suggested using HBA as an indirect sperm DNA assessment test to select the mature sperm for ICSI.[28] The present study provided even more scientific evidence that HBA reduces DNA fragmentation rate in semen processed with a combination of density gradient, swim up and HBA. Recently, Erberelli reported more than twice higher clinical pregnancy rate by using HBA, although not statistically significant.[18] Therefore, the available literature suggests that HBA selected sperm contain less DNA damage which may lead to better clinical outcome. More clinical research is required to confirm these findings.

In subgroup analysis, no difference in DNA damage rate was found between group A and B containing 2 and 3 abnormal semen parameters, respectively. Therefore, HBA could effectively improve sperm quality in patients with various sperm abnormalities.

We demonstrated that the HA binding process could achieve a three-fold reduction of DNA damage while previous studies demonstrated only two fold.[20] The use of HA binding viscous medium substituted the artificial PVP and had an obvious benefit in providing the selection of spermatozoa with intact DNA at the same time. On the other hand, this protocol could increase the cost of IVF treatment. Nevertheless, there was some limitation in our study. As all the samples were prepared and fixed for TUNEL assay, they could not be used for IVF treatment. Hence, no fertilization rate, embryo development or pregnancy rate could be observed and reported. Further research is needed to study the clinical outcome. Despite, human error may occur while using TUNEL assay because of the similar fluorescent color spectrum. In the future study, the assessment of DNA fragmentation using flow cytometry which is much more objective, should be adopted.

In conclusion, sperm preparation techniques could help decrease DNA damage in spermatozoa. The DNA fragmentation could be subsequently reduced even more by adding the HBA method to the conventional process of sperm preparation. All in all this study suggests that HBA method could be another choice for good sperm selection during ICSI.

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Conflict of Interest: The authors declared no conflict of interest.

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