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Comparison of the SSSperm testing kit with the Halosperm testing kit in an analysis of sperm DNA fragmentation

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Abstract:

The objectives of this work were to accurately evaluate testing kits that analyse sperm DNA fragmentation in infertile men and to provide a comparison of an improved testing kit (SSSperm testing kit) to the existing Halosperm testing kit in an analysis of sperm DNA fragmentation. A cross-sectional study was conducted on 300 semen samples from infertile men with a sperm concentration ≥1 million/ml using the Bland-Altman, T-test, and Pearson test for statistical study. The SSSperm testing kit had a coefficient of variation of CV%=2.26%<5% and t_{tn}=0.97<t_c=2. The two methods have similar DNA fragmentation index (DFI) results (r=0.995; p<0.001). The difference between the results of the two kits was not statistically significant (p=0.236>0.05). In conclusion, the SSSperm testing kit for the analysis of sperm DNA fragmentation is qualified as determined from quantitative tests, and the SSSperm testing kit is equivalent to the Halosperm testing kit.

Keywords: **comparison, DFI, improved test kit, infertile, sperm DNA fragmentation.**

Classification number: **3.2**

Introduction

Infertility is defined as the inability to achieve a clinical pregnancy after at least 12 months of regular unprotected intercourse [1]. Recently, infertility cases have quickly increased and has become a global health problem [2]. Globally, there are an estimated 15% of married couples affected by infertility and male infertility accounts for 30-40% of these cases [3, 4]. Male infertility can be initiated by testicular injury, sperm deficiency, or hormone problems [5], while one of the most prominent causes is sperm DNA fragmentation, which affects sperm function and male reproductive health [6].

Today, several methods of testing sperm DNA fragmentation exist such as COMET, TUNEL, SCSA, and SCD, but these methods require high-tech equipment, complex techniques, and high cost [7, 8].

In 2003, Fernández and partners proposed the sperm chromatin dispersion (SCD) test to determine sperm DNA fragmentation. This method is based on the principle that sperm without DNA fragmentation will form large halos around their nucleus, while sperm with DNA fragmentation will not produce halos or will produce very small halos around its nucleus when it is denatured in acidic environment and the nuclear protein is removed [9]. Based on this principle, Fernández and partners created the Halosperm testing kit in 2005 [10]. Since then, many studies of sperm DNA fragmentation using the SCD method or the Halosperm testing kit have been published and contributed significantly to the diagnosis and treatment of male infertility.

In Vietnam, some hospitals and research institutes have used the Halosperm kit to diagnose sperm DNA fragmentation, but the import cost of Halosperm is still high and thus not suitable for many patients. For this reason, our research team has built the SSSperm testing kit and evaluated the accuracy of the kit to determine the degree of sperm DNA fragmentation by the SCD method with the goal

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of completing the process and cutting the costs while still ensuring quality assessment of the degree of sperm DNA fragmentation in Vietnamese men. However, presently in Vietnam there does not exist a homemade kit that can both ensure the completeness as well as the accuracy of the level of sperm DNA fragmentation. Therefore, we conducted this research with the aim of evaluating the equivalency of the SSSperm testing kit and the Halosperm testing kit using the Bland-Altman, T-test, and Pearson tests.

Subjects and research method

Subjects

Three hundred semen samples from male patients who were diagnosed with infertility at the Hanoi Medical University Hospital were tested and assessed for sperm DNA fragmentation at the Genetic Counselling Centre, Hanoi Medical University Hospital.

The selection criteria for this study consisted of male patients aged from 18 years old whose semen analysis had a sperm density ≥1 million/ml and agreed to participate in the research.

The exclusion criteria for this study was male patients who do not meet the above criteria, have genital cancer, are infected with HIV, syphilis, or gonorrhoea, have an acute disease or mental illness, or did not agree to participate in the research.

Research methods

Sample size: to complete the procedure and determine the accuracy of this study, the following formula was used to calculate sample size for a descriptive research according to Lwanga and Lemeshow (1991) [11]:

$$n = Z^2_{1-\alpha/2}\,\frac{1-p}{\epsilon^2 p}$$

in which $Z_{(1-\alpha/2)}$: reliability coefficient (with 95% confidence, $Z_{(1-\alpha/2)}$=1.96); ε=0.10; p=95% (accuracy of reference procedure); n: number of required experiments, which was calculated to be 21 and was rounded up to 50.

To compare the SSSperm testing kit with the Halosperm testing kit, we used the following formula to calculate sample size:

$$n = Z^2_{\left(1-\frac{\alpha}{2}\right)} x\,\frac{p(1-p)}{(\varepsilon p)^2}$$

in which $Z_{(1-\alpha/2)}$: reliability coefficient (with 95% confidence, $Z_{(1-\alpha/2)}$=1.96); p=25% according to the research of Duran, et al. (2002) [12], where the rate of high sperm DNA fragmentation was >30% and had p=25%. For ε, we selected 0.2. Therefore, $n=1.96^2\times0.25\times(1-0.25)/(0.2\times0.25)^2=288.12$, which was rounded to 300. Thus, a sample size of 300 was used.

Research design: a cross-sectional study.

Method of making templates: the test (using the SSSperm testing kit) was an improvement of Fernandez, et al.'s SCD procedure (2005) [10] using the Halosperm kit from Halotech as follows:

Step 1. Preparation of agar: an agarose Eppendorf tube was placed into the float and melt using a water bath at 95-100°C for 5 min or in microwave for 3 min, until it was completely melted. The semen samples were diluted with a PBS solution such that the concentration of sperm was approximately <15 million/ml. The agarose tube was kept at 37°C for 5 min until the temperature of the Eppendorf-containing agar and of the incubator was balanced.

Step 2. Preparation of cell suspension: 25 µl of semen was added to an agarose tube and mixed well with a pipette. The tube was kept at 37°C while quickly moving on to the next step, in order to avoid solidification of the agarose. A drop of 25 µl of cell suspension was dripped on the circular position of the microscope slide, the slide was covered and gently pressed in order to prevent air bubbles from appearing. The slide was held horizontally throughout the entire process. The slide was placed in a refrigerator at 4°C for 10 min to allow the agarose to solidify.

Step 3. After the cell suspension was solidified, the slide was removed from the refrigerator and the microscope slide cover was removed by gently sliding it off of the slide. The denaturation of the sperm DNA was prepared by placing 80 µl of denaturing solution into a tube containing 10 ml of distilled water and shaking well. The slide with the sperm DNA was placed on the tray containing the denaturing solution for 7 min.

Step 4. Cell lysis: the slide was removed from the denaturing solution and placed in a tray containing 10 ml of lysis solution for 5 min.

Step 5. Wash the lysis solution: after finishing the lysis, the slide was placed in a tray containing distilled water for 5 min to wash off the lysis solution.

Step 6. Dehydration: the sample was dehydrated by adding the slide to an alcohol solution for 6 min, then allowing it to air dry.

Step 7. Dye the slide: the slide was placed horizontally and a Giemsa solution 5-30% was added dropwise to the surface of the slide. Then, it was left at room temperature for 10 min and washed with water from the tap. Excessively washing was avoided, which can lighten the halo colour.

Data processing

Evaluation of results: the microscope slide was observed under an optical microscope and at least 500 sperms were counted on the slide to determine the degree of sperm DNA fragmentation. Sperm DNA fragmentation was determined by sperm halo according to Fernandez, et al.

The rate of DNA fragmentation or DNA fragmentation index (DFI) was determined by the following formula:

$$\frac{sperms\ having\ small\ halo + sperms\ having\ no\ halo + degenerative\ sperms}{Total\ counted\ sperms} \times 100\%$$

Data analysis: to evaluate the accuracy of the SSSperm testing kit, two indicators were used: trueness and precision [13] (see Fig. 1).

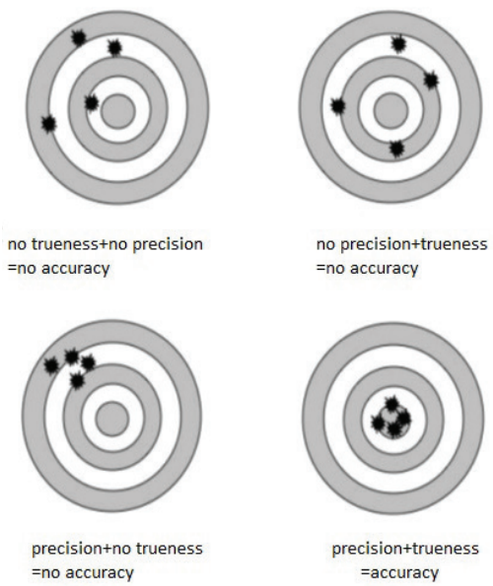

Fig. 1. Illustration of accuracy [13].

Precision is the degree of variation of independent test results around the mean. Precision is a qualitative concept and is expressed quantitatively by standard deviation or coefficient of variation. The lower the precision is, the larger the standard deviation or coefficient of variation. The formulae for standard deviation and coefficient of variation are, respectively,

$$SD=\sqrt{\frac{\sum(X_i-\overline{x})^2}{n-1}}$$

$$RSD\%=CV\%=\frac{SD}{\overline{X}}X100$$

in which SD: standard deviation; n: number of experiments; x_i: calculated value of the i^{th} experiment; $\overline{X}$: the mean value of the experiments; RSD%: relative standard deviation; and CV%: coefficient of variation.

Precision can be classified into four cases:

- Repeatability: repeatability expresses the degree of accuracy or repeatability, the degree of variation among experiment results which are done in the same laboratory with the same sample homogeneity, and by the same inspector over the same period of time. Repeatability is determined by the following method: on a patient's semen sample, an improved kit (SSSperm kit) is used to determine the degree of sperm DNA fragmentation and this is repeated 10 times. The standard deviation and coefficient of variation is calculated with a CV requirement ≤5%.

- Intermediate precision: the accuracy of the method is expressed according to the variables of laboratory. For several days, with different inspectors, and with different tools, the intermediate precision was found.

- Reproducibility: the accuracy of many laboratories conducting studies on the same homogeneous sample is expressed by reproducibility. Similar to repeatability, reproducibility is necessary provided that the laboratory or method is changed.

- Trueness: this indicates the degree of proximity between the mean of the experimental results and the real value, or accepted value.

Method to determine accuracy: on a patient's semen sample, from which the degree of sperm DNA fragmentation was determined with the Halosperm kit, the same experiment was conducted by using the SSSperm testing kit and repeated 10 times. The mean value and standard deviation were calculated, from which the standard, t_{tn}, was calculated using the following formula and then compared with Halosperm kit:

Công thức tính t_{tn}:

$$t_{tn}=\frac{|\mu-\overline{x}|}{\sqrt{\frac{s^2}{n}}}$$

in which: t_{tn}: experimental t value; μ: real value or accepted value (reference); $\overline{x}$: mean of experimental method; S^2: variance of experimental method; n: number of experimental times.

To compare the SSSperm testing kit with the Halosperm testing kit, the difference between the two tests was investigated based on Pearson correlation analysis, T-test, and a Bland-Altman plot using Epidata and SPSS.20 software.

Ethical research

All the patients' information was kept confidential and only analysed for fertility counselling for the patients and for this study, and not for any other purpose.

Results and discussion

Results

Accuracy evaluation of testing kit analysis of sperm DNA fragmentation in infertile men: a semen sample that had its DFI identified previously by using Halosperm kit, an improved kit (SSSperm kit) was used to determine the degree of sperm DNA fragmentation, and this was repeated 10 times. The results are in Table 1.

Table 1. Results of the test to determine the accuracy of the SSSperm kit.

Experiment	DFI (%)
1st	15.4
2nd	15.0
3rd	14.4
4th	14.2
5th	15.2
6th	15.2
7th	15.2
8th	15.0
9th	14.6
10th	15.0
Proof (made of Halo kit)	14.8

Precision: because the tests were conducted in the laboratory, we calculated the precision through repeatability. From the above results, Table 2 presents the results of the precision evaluation.

Table 2. Results of precision evaluation.

The mean of DFI (%)	14.92
SD	0.391
CV%	2.62%

In the experiments, especially during the quantitative tests, there are many errors that can affect the test and lead to inaccuracy of the results. Therefore, to control these confounding factors, it is necessary to use the concept of precision. The precision described in these results only depends on random errors and does not relate to the actual results of the sample. The lower the precision, the larger the standard deviation or coefficient of variation, otherwise, the greater the precision, the smaller the coefficient of variation is [13]. In this study, the SSSperm kit showed repeatability with a coefficient of variation CV%=2.62%. Therefore, the coefficient of variation had a value less than 5%, which, according to the Vietnam Standards [13] indicates that repeatability of this procedure meets requirements. Thus, when there are influences of random error factors for the same sample, the degree of sperm DNA fragmentation determined under different conditions has errors within the acceptable range.

Compared with the commercial Halosperm kit created by Fernandez, which has an actual coefficient of variation of 5.3% [14], the SSSperm kit has a lower coefficient of variation. This proves that the SSSperm kit meets the standards of a testing kit.

Trueness: the trueness indicates the degree of proximity between the mean values of the experimental results and the real values or accepted values. In the experiment to test trueness, we calculated t_{tn}=0.97. Besides, through searching tables, t_c=2.262 [13]. Thus $t_{tn}<t_c$. This means that the sperm DNA fragmentation index determined by the SSSperm kit has the same results as the commercial Halosperm kit and the process achieves the accuracy requirements of the analysis. Thus, the precision and trueness of the SSSperm kit completely meet the requirements of a testing kit according to Vietnamese Standards. This was the first step of the project.

Comparison of the SSSperm kit with the Halosperm kit: we have developed an improved procedure for determining the level of sperm DNA fragmentation, which is different from the Halosperm testing kit at the following key points (see Table 3).

Table 3. Improvements in techniques for testing sperm DNA fragmentation.

	Fernandez, et al. (2003) [9]	**SSSperm testing kit**
Denaturing solution	Denaturing solution of kit	HCl 0.29%
Lysis solution	Lysis solution 1: 0.4 M Tris-HCl; 0.8 M DTT; 50 mM EDTA; 1% SDS, pH 7.5. Lysis solution 2: 0.4 M Tris -HCl; 2 M NaCl; 1% SDS, pH 7.5	0.2 M Tris; 0.1 M DTT 2 M NaCl; 1% Triton, pH 7.5
Dehydration	3 steps with alcohol 70%, 90% and 100%	1 step with alcohol 100%
Dyes	Wright	Giemsa

After completing the SSSperm testing kit, we took 300 semen samples to make templates and assessed the degree of sperm DNA fragmentation by using the Halosperm testing kit and the SSSperm testing kit. The results are shown in the following chart (Fig. 2):

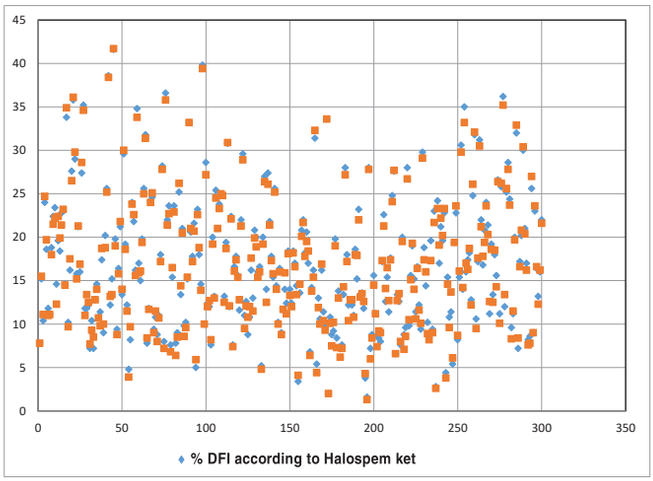

Fig. 2. Comparison of sperm DNA fragmentation rate determined by using Halosperm kit with SSSperm kit.

The value of the sperm DNA fragmentation index (DFI) measured by the Halosperm commercial kit and the SSSperm testing kit are almost similar. To compare the two kits quantitatively, we use the Pearson test, T-test, and Bland - Altman plot (Tables 4 and 5).

Table 4. Testing correlation coefficient between two kits.

N		**300**
Pearson correlation coefficient		0.995
P		**<0.001**
Confidence interval 95%	Upper limit	0.996
	Lower limit	0.994

The Pearson test shows a strong and significant correlation between the sperm DNA fragmentation index measured by the SSSperm testing kit and commercial Halosperm kit with r=0.995 and p<0.001.

Table 5. T-test table.

t	P	The mean of the difference	Confidence interval 95%	
			Lower limit	*Upper limit*
1.187	0.236	-0.010	-0.003	0.011

The results of the level of sperm DNA fragmentation assessment by the SSSperm kit and by the commercial Halosperm kit do not have statistically significant differences within a 95% confidence level (p=0.236>0.05).

The Bland-Altman plot is used to quantify the compatibility between two different measurements or to compare a new test with a standard recognized test. From the above tests, we have built a Bland-Altman plot showing the compatibility between measurement results of two tests (Fig. 3).

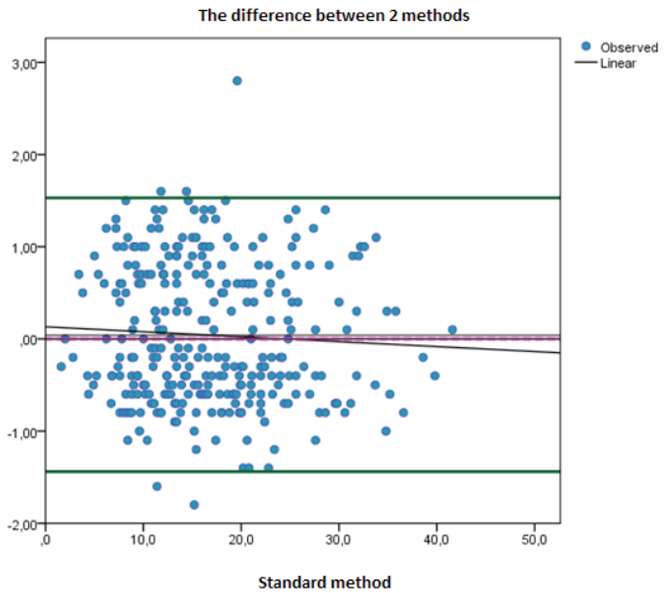

Fig. 3. Bland-Altman plot showing the compatibility of the two measurement methods.

The difference between the mean of the two kits is very small (0.042). Most cases have errors within the limit of ±1.96 standard deviations. Therefore, the SSSperm kit and commercial Halosperm kit have the same value in determining the degree of sperm DNA fragmentation.

Discussion

Sperms with fragmented DNA are unable to produce a halo of dispersed DNA loops while normal sperms succeed in producing the halo after treatment with a denaturing agent and removal of the nuclear proteins. Based on this principle, we created an improved test (using the SSSperm kit) to determine sperm DNA fragmentation.

There are advantages of the SSSperm kit and notable differences between the improved test and other existing tests. For example, the improved test is a quantitative test. Unlike semi-quantitative tests like COMET and TUNEL, which determine sperm DNA fragmentation by colour and fluorescence intensity, the improved test determines sperm DNA fragmentation by measuring the percentage of sperms with non-dispersed (have no halo or small halos) or dispersed DNA loops (have large halos), which can be observed with the naked eye.

The Halosperm testing kit is also based on the principle that sperm with fragmented DNA fail to produce halos while normal sperm produce large halos, which was published by Fernandez, et al. in 2003. There have been some studies conducted to evaluate the value of this kit [9]. The results obtained from these studies indicated that this testing kit meets the accuracy requirement to

determine sperm DNA fragmentation and thus it has been widely used in diagnosing male infertility, especially in Vietnam. However, the price of this kit is still high, which is not suitable for many Vietnamese citizens. Therefore, we created an improved testing kit (SSSperm testing kit) which is simpler and cheaper than the Halosperm testing kit but still ensures quality results. When the Pearson test, T-test, and Bland-Altman plot was used to compare the SSSperm testing kit with the Halosperm testing kit, the results indicated that there were significant correlations between the two kits ($r=0.995$, $p<0.001$) and the mean of difference was -0.01, $p=0.236>0.05$, therefore the difference was not statically significant.

In conclusion, the improved test is accurate, fast, inexpensive, and simple. Therefore, the SSSperm testing kit should be used as a routine kit in Vietnam to determine sperm DNA fragmentation in infertile men.

Conclusions

The SSSperm testing kit has the required accuracy of a quantitative testing kit (with CV%=2.62%<5% and $t_{tn}=0.97<t_c$).

The results obtained from the improved kit is equivalent to the commercial Halosperm kit. Differences in the results obtained from the two methods are not statistically significant and are completely random.

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The authors declare that there is no conflict of interest regarding the publication of this article.

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