[Original Article]

Evaluation of the sperm DNA fragmentation index in infertile Japanese men by in-house flow cytometric analysis

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Abstract

Semen analysis has long been used to evaluate male fertility. Recently, several sperm function tests have been developed. Of those, the sperm DNA fragmentation index (DFI), which describes the status of the sperm DNA, is thought to be a suitable parameter for evaluating male fertility. However, there have been no large-scale studies on the sperm DFI of Japanese men. Therefore, we investigated the feasibility of using an in-house flow cytometry-based sperm DFI analysis based on the Sperm chromatin structure assay (SCSA) sperm DNA fragmentation test to assess male fertility in Japan. This study enrolled 743 infertile and 20 fertile Japanese men. To evaluate reproducibility, inter- and intra-observer precision were analyzed. A receiver operating characteristic curve analysis was used to set a cut-off value for the sperm DFI to identify men who could father children by timed intercourse or intrauterine insemination. The variability of the sperm DFI among fertile volunteers was determined. The relationship between semen parameters and the sperm DFI was assessed by Spearman's rho test. A precision analysis revealed good reproducibility of the sperm DFI. The cut-off value of sperm DNA fragmentation in infertile men was 23.95%. Semen volume had no relationship with the sperm DFI. Sperm concentration, sperm motility, total motile sperm count, and percentage of normal-shaped sperm were significantly and negatively correlated with the sperm DFI. The median sperm DFI was smaller in fertile volunteers (7.70%) than in infertile men (19.37%). Sperm DNA fragmentation analysis can be used to assess sperm functions that cannot be evaluated by ordinary semen analysis.

Key words: semen analysis; DNA fragmentation; male infertility; spermatozoa; flow cytometry

Introduction

Semen analysis, such as investigation of semen volume, sperm concentration, sperm motility, and sperm head morphology, has long been used to evaluate male fertility. However, in the era of modern assisted reproductive technologies (ARTs), such as *in vitro* fertilization and intracytoplasmic sperm injection, several test of sperm function have been developed, including the zona-free hamster egg sperm penetration test [1] and sperm motion parameter measurement.[2] The most important task of sperm in reproduction is to transport genetic information to the egg. Therefore, the status of sperm DNA has been suggested as a suitable parameter for evaluating male fertility.[3] The integrity of sperm DNA can be assessed by screening sperm for DNA fragmentation to provide a measure of the proportion of damaged sperm within an ejaculate; this sperm DNA fragmentation index (DFI) has been used in investigations of male fertility in several countries;[4-9] however, no large-scale investigation of the sperm DFI of Japanese men has been performed to date. Significant differences in sperm quality have been identified between male populations, even between geographically close countries: for example, sperm quality in Finland is higher than that in Denmark.[10] Here, we sought to establish a standard value for DFI in Japanese patients and also reconfirmed the necessity of measuring DFI in a clinical setting.

In this study, we first set up an in-house flow cytometry-based sperm DNA fragmentation analysis system and evaluated the reproducibility of the assay. Second, we measured the sperm DFI of infertile Japanese men and evaluated its correlation with standard semen parameters. Third, we identified a cut-off value of sperm DFI that could predict which men could achieve fatherhood without the use of modern ART.

Materials and Methods

Patients

Initially, 1310 Japanese male patients attending our institution and two affiliated clinics (Umegaoka women's clinic in Tokyo, Japan and Tsubaki women's clinic in Matsuyama, Japan) between April 2017 and April 2018 due to infertility were screened for inclusion in this study. All were screened at our institution; those whose wives had infertility factors were excluded. Patients with ejaculatory or erectile dysfunction and those with malignant disease or chronic illness (e.g., end-stage renal disease or liver dysfunction) were excluded. Also excluded were patients with azoospermia, those with a sperm concentration less than 300,000 sperm/mL, and those who provided semen samples after less than 2 days or more than 5 days of abstinence. Sixty patients, whose sperm DFIs were measured without snap freezing, were also excluded. Although the data reported by Evenson & Jost indicate that the DFI does not change significantly between fresh and snap-frozen samples,[11] it is intended that DFI measurement in the central laboratory of Japan will be performed using frozen samples as some infertility clinics cannot afford the cost of running a flow-cytometer. Thus, we excluded fresh samples in the present study. In total, 743 patients were included in this study. Between September 2017 and December 2019, a total of 130 patient couples who had tried to conceive by timed intercourse (i.e., having intercourse on the day selected according to ovulation

monitoring) or by intrauterine insemination (IUI) for at least 6 months were enrolled in the study. Pregnancy was confirmed by the detection of a fetal heart beat by ultrasound. In this study, couples in which the wife had gynecological problems or was aged over 40 years were excluded. We measured the DFI at every visit. The DFI value adopted was the latest one before confirmation of pregnancy.

Fertile volunteers

Twenty men whose wives had conceived without medical intervention and delivered children within the 6 previous months were asked to produce semen samples after 2–3 days of abstinence. All participants were Japanese.

Informed consent

All participants were informed of the study protocol and provided written informed consent. All procedures conformed to the provisions of the Declaration of Helsinki. This study was approved by the institutional review board of Dokkyo Medical University Saitama Medical Center in Koshigaya, Japan. (Approval No. 2075)

Semen analysis

Semen collection and analysis were performed according to the method recommended by the World Health Organization 2010 guidelines with slight modification.[12] Semen was collected in a sterile container and allowed to liquefy completely at room temperature. The ejaculate volume was estimated by sample weight assuming a density of 1 g/mL. At first, sperm concentration was assessed using Makler’s Chamber (Sefi Medical Instruments, Ltd, Hifa, Israel) on fresh semen samples to obtain an

approximate estimate of sperm numbers. Later, sperm concentration was determined by counting immobilized sperm in a fixed sample using the improved Neubauer hemocytometer (Hawksley, Lancing, United Kingdom). Sperm motility was measured as the percentage of spermatozoa showing progressive and non-progressive motility (total motility). To evaluate sperm morphology, 10 μL of the liquefied semen was spread onto a glass slide and air-dried. After Diff Qick® staining (Dade Behring Inc., Newark, NJ, USA), the slides were examined at ×400 and ×1000 magnification under a light microscope (BX43; Olympus, Tokyo, Japan). The proportion of normal-shaped spermatozoa was determined by assessing at least 300 spermatozoa according to strict criteria.[13] Since the recognition of abnormal sperm morphology depends on subjective criteria, one of the authors (TT) was assigned to perform the morphological evaluations. In this study, sperm morphology in 124 samples was evaluated by the procedures described above.

Sperm DFI measurement

Semen samples were snap frozen in liquid nitrogen and stored at -80°C until use. Frozen semen samples were thawed at 35°C in a water bath. The sperm DFI was evaluated by the sperm chromatin structure assay (SCSA) as described by Evenson et al.[4] Briefly, semen (10 μL) was diluted with Tris-NaCl-EDTA buffer (90 μL). The suspension was treated with 200 μL of pH 1.2 buffer for 30 s to denature the DNA at the sites of strand breaks. Subsequently, 600 μL acridine orange staining solution (0.0006% v/v in phosphate citrate buffer) was added, and the sample was placed in a CYTOFLEX flow cytometer interfaced with CytExpert Version 2.3 software (Beckman Coulter, Inc., Atlanta, GA, USA). All samples were independently measured twice, and at least

20,000 events were analyzed per measurement. Single-stranded DNA fragments combined with acridine orange emit red fluorescence, while intact double-strand DNA combined with acridine orange emits green fluorescence. The proportion of sperm with red fluorescence within the total number of spermatozoa was calculated to obtain the sperm DFI. The flow cytometric data were analyzed using dedicated software (Beckman Coulter, Inc., Atlanta, GA, USA). Before measurement, reference samples were run to verify the performance of the measuring system. The reference samples came from the stored samples of healthy donors and patients with oligoasthenoteratozoospermia whose sperm DFI values had been measured previously. Two of the authors (TT and SO) measured all samples.

Precision assay

The reference semen samples containing 2.0×10^6, 20.0×10^6, and 100.0×10^6/mL sperm were prepared and stored in 50-μL aliquots until use. The samples were measured on 10 separate days, and the coefficients of variation (CVs) of the measured sperm DFIs were calculated for each sample as between-run precision. To test the consistency of the results, repeated measurements of the samples were performed 10 times. The precision of each measurement was assessed by the CV obtained as within-run precision.

Statistical analysis

Statistical analyses were carried out using SPSS Statistics for Windows, version 26 (IBM Japan, Tokyo, Japan). After checking the normality of the data, a nonparametric test (Spearman’s rank correlation) was used to analyze the correlations between semen parameters and the DFI values. A receiver operating characteristic (ROC) curve analysis

was used to obtain the cut-off value of the sperm DFI to differentiate men who could father children by timed intercourse or IUI from those who could not. Then, the sensitivity and specificity of the optimal cut-off value were assessed. A *P* value of less than 0.05 was considered statistically significant.

Results

Between- and within-run precision

Between-run precision was analyzed using three reference samples measured on 10 separate days. The CVs were 3.9%, 2.2%, and 1.8% for measurements of semen containing 2.0×10^6, 20.0×10^6, and 100.0×10^6/mL sperm, respectively. To test the consistency of the results, the measurements were repeated 10 times by two of the authors. The CVs of measurements by TT and SO were 2.5% and 2.3%, respectively.

Sperm DFI and semen parameters

In total, 743 samples from infertile men attending a male infertility clinic were used to measure the sperm DFI by an in-house flow cytometry-based method. The values of the measured sperm DFIs were distributed widely: median 19.37% (range, 1.18–92.73%). We analyzed the correlations between the sperm DFI and semen parameters by Spearman's rho test, and the results are shown in **Table 1 and Figure 1**. The analysis showed that the sperm DFI was negatively correlated with sperm concentration, sperm motility, total motile sperm count, and percentage of normal-shaped sperm (all $P < 0.001$). Semen volume had no relationship with sperm DFI.

Cut-off value of sperm DFI

Sperm DFI data from 130 patient couples who tried to conceive by timed intercourse or IUI were used to determine the cut-off value of the sperm DFI. The characteristics of semen analysis and DFI measurement in 130 patients was shown in Supplementary Table 1-2. A ROC analysis of DFI in patients who did not achieve a pregnancy by timed intercourse or by IUI compared to those who succeeded in pregnancy is shown in **Figure 2.** A DFI cut-off value of 23.95% showed the highest sensitivity and specificity for distinguishing fertile patients from infertile patients. When the threshold level 23.95% was used, sensitivity and specificity were estimated as 0.816 and 0.728, respectively. The area under the curve was 0.842.

Sperm DFI in proven fertile volunteers

Sperm DFI values were measured using semen samples from 20 volunteers whose wives had conceived without medical intervention. The men provided semen samples within 6 months after delivery. Values of semen parameters and the distribution of sperm DFI values are shown in **Table 2**. The median sperm DFI value of the fertile volunteers was 7.70% (standard deviation, 3.2%; range, 3.18–17.3%).

Discussion

Standard semen analysis has been used for several decades to evaluate male fertility. However, comparison of semen parameters between fertile and infertile men has revealed the presence of large overlaps in these parameters.[14] After the introduction of modern ART, such as *in vitro* fertilization and intracytoplasmic sperm injection, the demand for a sperm function test that can be used to select appropriate ART has

increased rapidly.[15] For the process of fertilization, the most important role of spermatozoa is to transport genetic information to the oocyte. Thus, the status of DNA in the sperm head should be prioritized over the evaluation of other sperm functions.[16-19] There are several methods for measuring DNA fragmentation in spermatozoa.[20] The most widely used are terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labeling (TUNEL),[21] the sperm chromatin dispersion test,[22] the alkaline comet assay,[23] and the SCSA.[4,24] The two flow cytometric assays (TUNEL and SCSA) have the highest reliability and reproducibility for determining the sperm DFI, and comparison studies have shown an association between the results obtained from these two methods.[25]

We selected the SCSA, which was established through the pioneering work of Evenson et al.[26] This method has clear advantages over other approaches as it is possible to freeze samples at -80°C, making it possible to collect numerous samples before analysis, and the measurement protocol is relatively easy.[4, 27] The SCSA® has been commercialized since 2005 and centralized to large diagnostic centers with a license from the SCSA® group. However, recent advances in flow cytometry have made it possible to establish in-house flow cytometric analysis of sperm DNA fragmentation.[27] The protocol adopted in this study was based on the one described previously.

To evaluate the reproducibility of the results from our in-house assay, we conducted between- and within-run precision analyses. The protocol used in this study showed high reproducibility with CV values of less than 4%. These results demonstrate the high reproducibility of the in-house flow cytometry-based sperm DFI assay.

Although the relationship between DNA damage and semen parameters is well established in the literature, there is relatively little specific information on DNA

damage in semen samples from Japanese men. The present study is the first to provide data on DNA damage and semen parameters from a large sample of Japanese men. With one exception,[28] the published studies indicate that spermatozoa from patients with abnormal semen parameters have increased levels of DNA damage.[3, 20, 29] In the present study, Spearman's correlation analysis showed that the sperm DFI correlated negatively with sperm concentration, sperm motility, total motile sperm count, and percentage of normal-shaped sperm. Semen volume showed no relationship to the sperm DFI. These results are consistent with those of previous studies.[3, 5, 6, 19, 20, 30]

In a 6- to 24-month follow-up period, 130 patient couples tried to conceive by timed intercourse or IUI. From a ROC analysis, we determined a cut-off value of 23.95% for the sperm DFI to predict fecundity. This threshold of 23.95% DFI is very similar to the 25% threshold reported by Evenson et al.[4] This is the first attempt to set a cut-off value for Japanese patients who seek to conceive children by timed intercourse or IUI. This value can be used when deciding an appropriate treatment strategy for infertile Japanese couples.

Our next objective was to identify the normal statistical limit of the sperm DFI. For this purpose, we asked 20 volunteers who had fathered children without medical intervention to provide semen samples. The median sperm DFI value of these volunteers was 7.70%, which was much lower than the cut-off value determined above. By contrast, the median sperm DFI of infertile men attending a male infertility clinic was 19.37%. This suggests that male infertility patients may have some underlying causes for their raised sperm DFI values. However, the number of fertile volunteers in this study was relatively small. A cohort study with a larger number of volunteers will be necessary to confirm the median sperm DFI value of fertile Japanese men.

Sperm DFI measurement can be used to evaluate sperm function and to monitor the effectiveness of treatments. To establish DFI values and their impact on the management of infertility, a large-scale study of the correlation between the sperm DFI and the outcome of infertility treatment, with live births as an end point, is warranted. In addition to sperm DFI value, the proportion of sperm with DNA stainability (%HDS) due to retained histone complexed DNA can be measured simultaneously.[24] Previous studies suggested that the level of HDS sperm is negatively correlated with pregnancy success.[31,32] We did not perform HDS analysis in this study, and further research is needed to elucidate the clinical implications of these values.

Author Contribution

Design of the study was conceived and manuscript was prepared by AO. Acquisition and analysis of the data was done by TI and YS. Semen analysis and DFI measurement was done by SO and TT. Data analysis and interpretation was performed by YK. Design of the study was prepared, data analysis and interpretation were done, and the revised manuscript was drafted by HO, KS and KS. All authors approved the final version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

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Table 1. Correlations between the sperm DFI and semen parameters

Semen parameter	Mean± SD	Correlation coefficient with sperm DFI (r)	P value	Evaluated number of samples
Semen volume (ml)	2.90±1.47	-0.011	0.597	n=743
Sperm concentration (10^6 per ml)	45.34±50.57	-0.416	<0.001	n=743
Total motility (PR+NP, %)	31.76±23.07	-0.413	<0.001	n=743
Total motile sperm number X10^6	46.71±90.91	-0.465	<0.001	n=743
Sperm morphology (normal forms, %)	4.72±3.16	-0.378	<0.001	n=124

Correlations between sperm DFI and semen parameters were analyzed by Spearman's rho test.

DFI, DNA fragmentation index; SD, standard deviation; PR, progressive motility; NP, non-progressive motility.

Table 2 Seminograms and sperm DFI of 20 fertile volunteers

Parameters	Median (SD)	Minimum	Maximum
Age (years)	33 (4.2)	28	41
Abstinence (days)	3 (0.5)	2	3
Semen volume (ml)	2.9 (1.49)	1	6.4
Sperm concentration (10^6 per ml)	66.5 (42.6)	22	159
Total motility (PR+NP, %)	55.5 (14.79)	31.6	81.1
Sperm morphology (normal forms, %)	4 (1.0)	3	6
DFI (%)	7.70 (3.2)	3.18	17.30

The sperm DFI was measured using semen samples provided by 20 volunteers whose wives had conceived without medical intervention.

DFI, DNA fragmentation index; SD, standard deviation; PR, progressive motility; NP, non-progressive motility

Supplementary Table 1: The characteristics of semen analysis and DFI measurement in 81 patient couples who achieve pregnancy by timed intercourse or IUI

Semen volume (ml)	Sperm concentration (106 per ml)	Total motility (RP+NP,%)	Sperm morphology (normal forms, %)	DFI (%)
1.8	157.7	94	3.7	3.55
5	96.3	74	2	4.98
3.6	18.3	86	4.5	6.54
1	79.8	25	2	6.76
2.9	41.2	80	3.7	7.59
2.4	32.9	61	0	7.66
1.7	61.9	84	0	7.84
2.5	50	51	3.3	8.04
3	44.4	61	3.3	8.25
3.2	38.8	76	1.3	8.43
4.8	106.1	89	3	8.72

1.8	24.7	73	1.7	8.83
2.2	56.3	84	3.3	9.11
4.2	332.1	88	0.3	9.28
4	119.1	89	2.6	9.47
2.6	70.5	81	5.7	10.39
1.6	70.5	79	2	10.59
3	69.1	55	2.7	10.64
2.3	37	64	5	10.82
2	29.8	53	4	11.10
4.1	28.4	73	0.3	11.59
1.5	5.9	41	0.6	11.67
2	274.4	65	1.3	12.00
4.1	26.3	79		12.38
1.5	38.3	81	1	12.49
4.2	35.7	64	1.3	13.40

1.8	192.3	72	3	13.46
1.2	58.5	93	3.3	13.60
2.5	17.1	36	1.3	13.84
2.9	93.8	51	1	14.01
2.6	36.4	29	1	14.33
5.8	62.5	57	1.3	14.88
4.5	20.9	46	2	15.01
3.8	54.3	68	5	15.15
2.2	33.7	20	1.7	15.20
1.2	141	93	3.3	15.25
3.7	52.1	53	0.7	15.57
4.9	69.2	79	4.6	15.93
1.5	44.6	62	1	16.01
1.5	59.5	66	1	16.18
2.6	27.6	40	1.3	16.31

3.6	67.3	65	1.3	16.35
0.9	92.8	74	2.3	16.42
2.8	26.8	48	1.3	16.70
1.8	89.6	17	3	16.81
0.9	185.9	74	4	17.12
3	27.7	69	4.1	17.81
3.4	79	81	3	18.18
5.4	29.3	83	2.7	19.18
6.8	37.8	68	2	19.64
3.5	63.3	88	2.7	19.76
6.1	21.8	75	3.3	19.95
2.6	17.6	62	4.3	19.98
3.4	19.6	25	5.8	20.65
3.9	35.3	45	2	21.84
1.6	50.2	64	1.3	23.02

3.9	35.6	59	1.7	23.16
5.3	83.7	57	2.7	23.73
2	18.9	61	3	23.78
3.2	15.5	44	1	24.56
2.5	19.9	33	1.7	27.10
1	27.1	41	0.7	28.41
2.9	35.3	44	0.7	28.85
5.4	13.8	57	0.7	30.08
3.6	74.2	52	1.3	30.61
4	56.3	59	1.3	30.64
5.2	29.3	73	1.6	30.80
4	149	74	3.3	30.91
3.1	111.9	33	0.7	32.16
2.1	85.4	57	3.7	32.62
4.2	22.1	68	3.3	33.31

0.8	23.7	38	0.6	33.70
2.4	28.5	51	3	34.31
3.5	58.8	88	1	35.75
5.4	41.8	87	4.3	38.24
4.1	37	86	4	39.82
1.9	204	3	0.7	42.31
6.6	19.4	89	3.3	42.83
5.3	25.5	60	3	43.81
2.8	35.9	73	1	48.84
5.4	20.5	4	1	50.52

DFI, DNA fragmentation index; PR, progressive motility; NP, non-progressive motility

Supplementary Table 2: The characteristics of semen analysis and DFI measurement in 49 patient couples who did not achieve pregnancy by timed intercourse or IUI

Semen volume (ml)	Sperm concentration (X106/ml)	Sperm motility (%)	Normal morphology (%)	DFI (%)
2.1	3.8	33	2.3	11.60
2.6	12	80	1	11.71
3.2	8.3	67	1.3	16.99
5.1	11.2	37	1.4	17.14
5.3	19.1	58	0.3	18.78
3	72.4	73	0.7	19.20
2	9.1	40	1.3	20.28
3.6	67.8	56	2.3	23.07
3.6	29.6	59	5.3	23.29
1.1	14.4	12	2.7	24.13

5.1	15.4	56	0.3	24.48
0.9	27.9	40	2.3	24.74
no data	no data	no data	5.2	25.08
2.6	30	21	0.3	25.57
1.8	74.2	79	1.7	26.39
6.3	21.5	45	0.3	29.54
1.2	80.6	87	4.7	30.03
6	6.1	39	0.7	30.32
2.2	4.8	33	4.6	30.82
4	9	48	1.3	31.06
4.8	3.4	57	6.9	31.91
2.9	8	34	3.3	32.02
4.3	7.4	20	0.3	33.47
8.9	14.6	40	0.3	33.52
1.9	2.1	31	2.2	34.06

5.1	3.4	76	1.3	34.61
0.4	11.7	26	0.3	35.59
3.8	13.8	52	0.3	39.98
4.7	68.8	65	3.3	40.80
3.4	7.1	27	3.5	43.93
0.8	3.8	21	0.3	47.25
3.8	4.2	40	5.4	49.99
2	2.4	27	2	51.33
3.2	1.4	44	0.7	51.54
3.5	12.7	52	2	52.13
2.2	15.1	26	0.3	52.38
2.4	44.2	18	0.3	53.82
1.5	90.6	11	1	54.66
4.4	8	44	1.3	56.13
2.9	2.2	36	0.7	57.68

4.4	2.9	33	2.7	59.30
3	13.3	23	1.3	60.74
3.5	17.5	87	0.3	62.54
1.7	6.9	7	1	63.33
5.9	13	86	4.7	63.89
6.8	3.7	43	0.3	63.91
3.1	27.9	90	3.7	65.35
2.9	2.9	44	0.6	67.96
1	2.7	29	0.3	75.62

DFI, DNA fragmentation index; PR, progressive motility; NP, non-progressive motility

Figure legends

Fig. 1: Correlations between semen parameters and the sperm DNA fragmentation index (DFI). a, Semen volume had no relationship with the sperm DFI. b–e, Sperm DFI was negatively correlated with sperm concentration, total motility, total motile sperm count, and sperm morphology.

Fig. 2 Receiver operating characteristic curve of the sperm DNA fragmentation index. A cut-off value of 23.95% was set to differentiate fecundability.