

Frequency of Sperm DNA Fragmentation According to Selection Method: Comparison and Relevance of a Microfluidic Device and a Swim-up Procedure

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ABSTRACT

Introduction: Multiple rounds of centrifugation or washing spermatozoa can cause sperm DNA fragmentation (SDF); however, a microfluidic approach to select spermatozoa does not require centrifugation. Reports have suggested that sperm sorting using a microfluidic device is an effective method to select good quality spermatozoa, however, it is not known whether it reduces sperm DNA damage. We investigated whether the frequency of SDF was affected by selection method during sperm processing.

Materials and Methods: Semen samples from ten men with normal, oligozoospermia and asthenozoospermia were split into

two groups and sorted using a microfluidic device or by a swim-up method. Subsequently, semen parameters and SDF were measured and analyzed using paired or non-paired Student's *t*-tests.

Results: For samples sorted by the microfluidic device (Sperm Sorter Qualis[®]; Menicon, Kasugai, Japan) or the swim-up method, both showed a decrease in SDF. However, the decrease was more significant when the microfluidic device was used.

Conclusion: Sorting using the microfluidic device resulted in less SDF than did the swim-up method.

Keywords: Sperm concentration, Spermatozoa, Sperm selection

INTRODUCTION

In assisted reproductive technology (ART), the diagnosis of male infertility has been conducted based on the assessment and analysis of sperm concentration, motility and morphology with the aim of obtaining the best quality of spermatozoa. Any type of damage present in sperm DNA can lead to ART failure [1]. Sperm DNA fragmentation might be the most frequent cause of paternal DNA anomaly transmitted to progeny, as it is found in a high percentage of spermatozoa in subfertile and infertile men; such fragmentation is negatively correlated with semen quality [1].

Sperm DNA fragmentation (SDF) is being recognized increasingly as a critical issue for male infertility. However, there are only a few techniques to identify this in routine laboratory use. Popular techniques for selecting good spermatozoa for intracytoplasmic sperm injection (ICSI) include a swim-up from either raw semen or a washed pellet, and density-gradient centrifugation [2,3]. These techniques have been used to separate functional spermatozoa suitable for use in conventional in vitro fertilization (IVF) and ICSI [4]. However, single or multiple centrifugation steps damage spermatozoa via the generation of reactive oxygen species (ROS) [5,6]. SDF can be induced by various factors such as apoptosis, ROS, chemotherapy and radiotherapy, activation of caspases and endonucleases, and by environmental toxicants [7]. SDF produces adverse consequences during postimplantation development of the embryo rather than before it [8]. Therefore, selecting spermatozoa without SDF might be essential for ART success.

A device based on microfluidic flow theory (Sperm Sorter Qualis[®]; Menicon, Kasugai, Japan) has been shown to select morphologically normal spermatozoa with high motility and with no debris [9-12]. As centrifugation is not necessary for this device, it is possible that it could isolate highly motile spermatozoa with a low frequency of SDF. However, although studies have demonstrated the clinical efficacy of this microfluidic device for selecting motile spermatozoa, there is scant information regarding the effect of such devices on DNA damage during semen processing for ART. Therefore, we compared the frequency of SDF after using the microfluidic device

with that of raw semen, and with spermatozoa sorted by a swim-up procedure.

MATERIALS AND METHODS

The Sperm Sorter Qualis[®] [Table/Fig-1] is a commercially available device based on microfluidic theory and is designed to have a channel in which nonmotile sperm and debris are obliged to flow along the initial stream and arrive at a chamber, D. Motile spermatozoa migrate to another channel, overcome the boundary of two streams and accumulate at a collecting chamber, C. Fresh semen samples obtained from 10 men (mean age 37.4 years, range 30–48; [Table/Fig-2]) with normal, oligozoospermia and asthenozoospermia were split into two groups and sorted either using Sperm Sorter Qualis[®] or by swim-up. Routine semen analyses were performed according to the World Health Organization (WHO) guidelines [13].

For using the Sperm Sorter Qualis[®], semen samples were diluted 1:1 in Universal IVF Medium[®] (Origio, Malov, Denmark), filtered to remove debris and then kept at 37°C until used. The device was fixed on the bottom of a 60-mm plastic dish, and 100 µl of washing medium was pipette into chambers A, B, C and D, and retrieved. Then 65 µl aliquots of diluted spermatozoa were loaded into chamber A at room temperature [Table/Fig-1]; 100 µl of medium without semen was loaded into chamber B, and 20 µl of medium was loaded into each of chambers C and D. The volume of medium in chamber B was adjusted to be 40% of the whole width of the device [Table/Fig-3]. Spermatozoa in the medium in chamber C were retrieved 30 min after samples had been loaded and subjected to analysis and examination of DNA fragmentation.

A separate swim-up method was performed as follows [6]. Briefly, diluted semen was layered onto 50% Percoll[®] (GE Healthcare Life Sciences, Pittsburg, PA, USA), 50% Universal IVF medium[®] and centrifuged for 20 min at 652g at room temperature. The supernatant was removed and pellet was resuspended with 1 ml of medium, and centrifuged for 5 min at 163g. The pellet was used for swim-up between 5 to 30 min after removing the supernatant and debris.

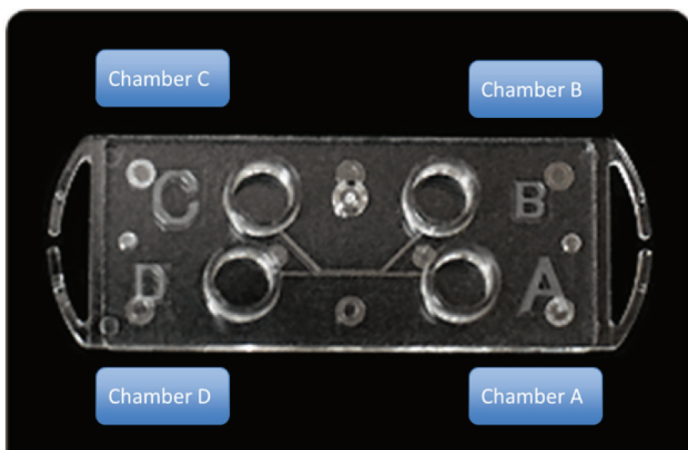
SDF was assessed using Halosperm® kits (Halotech DNA SL, Madrid, Spain) according to the instructions of the company. Glass slides with samples were stained with Diff Quik® (Dade Behring, Deerfield, IL, USA) and 300 spermatozoa per slide were observed in each sample for evaluating SDF. SDF was shown as the percentage.

STATISTICAL ANALYSIS

Data are shown as the mean±SD. Parametric data were analyzed statistically using Student's *t*-tests. Paired tests were used for comparing data before and after sperm separation and non-paired tests were used for comparing the Sperm Sorter Qualis® and swim-up group. The software used for statistical analysis was StatMatelll (Human Body, Tokyo, Japan) and *p*<0.05 was considered significant.

RESULTS

The initial sperm counts from three of the subjects were more than 50×10⁶/ml with high motility [Table/Fig-2]. For three others, the counts were less than 15×10⁶/ml with high motility of over 40%, and the remaining four men had counts of more than 15×10⁶/ml with motility of less than 40%. The rates of morphological abnormalities and linearity of sperm movement were similar in these three groups.



[Table/Fig-1]: Illustration of the microfluidic device, Sperm Sorter Qualis®. An aliquot of semen is loaded into chamber A at room temperature, whereas medium without semen is loaded into chamber B. Spermatozoa with good quality are retrieved from chamber C.

	Sperm numbers (x10 ⁶)	Motility (%)	Abnormal Morphology (%)	Linearity (%)
1	83	53	30	60
2	75	72	30	70
3	57	61	25	60
4	7.4	46	30	50
5	13.6	41	60	50
6	3.2	94	30	50
7	37	32	25	60
8	19	37	30	55
9	43	26	35	60
10	52.5	20	45	45

[Table/Fig-2]: Semen analysis of samples in initial samples

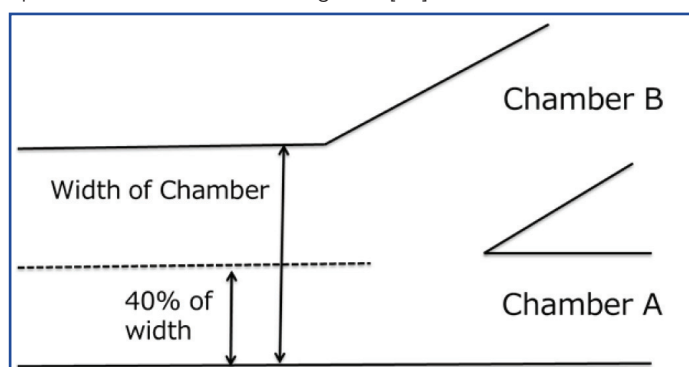
The percentage of SDF in the initial semen samples was 27.7% and in the post-centrifugation samples it was equivalent to the initial samples (25.8%; [Table/Fig-4]). SDF decreased to 8.3% after swim-up preparation (paired *t*-test, *p*=0.0005; [Table/Fig-4]) and to 5.9% in samples sorted by the Sperm Sorter Qualis® (paired *t*-test, *p*=0.0001). Similar findings were evident when DNA fragmentation of normal individuals was compared with a low motility group and a low sperm count group (data not shown).

When the percentage of SDF was compared between the swim-up and Sperm Sorter Qualis® results, that of the microfluidic device was significantly lower than swim-up (nonpaired *t*-test, *p*=0.0002, [Table/Fig-5]).

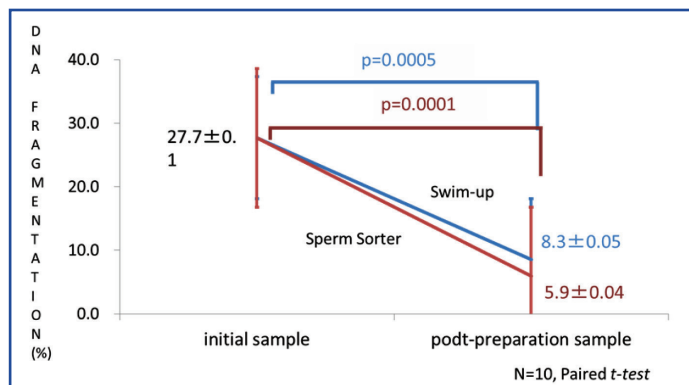
DISCUSSION

We found that motile spermatozoa prepared by the Sperm Sorter Qualis® showed reduced SDF compared with presorted samples and samples prepared by swim-up, which is a common method for sperm selection in ART.

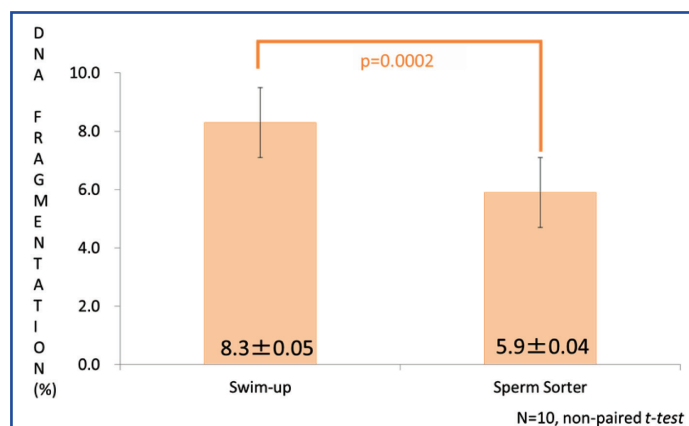
Selection of the best spermatozoa and elimination of damaged spermatozoa are critical for successful IVF and ICSI in infertility clinics. At the present time, the most prevalent methods for isolating good spermatozoa are density gradient separation by the Percoll® and swim-up methods. However, one or two centrifugation steps are required to separate spermatozoa in both methods, and this might contribute to damage [14]. Furthermore, sperm damage by centrifugation might lead to increased levels of ROS causing SDF [15]. However, this microfluidic device is able to select good spermatozoa without centrifugation [10]. This should cause much



[Table/Fig-3]: This illustration shows adjustment of liquid volume in laminar flow. Volume of medium in chamber B is adjusted to 40% width to the whole chamber width



[Table/Fig-4]: The percentage of sperm DNA fragmentation (SDF) before and after preparation by swim-up or Sperm Sorter Qualis®. The percentage of SDF decreased significantly with both techniques



[Table/Fig-5]: Comparison of SDF percentages between the swim-up and Sperm Sorter Qualis® preparations. That in the latter group was significantly lower than after swim-up

less stress to spermatozoa leading to the selection of good quality spermatozoa. The advantages of this device are that it can select highly motile and morphologically normal spermatozoa from semen samples and because they show less SDF for use in ART procedures [10-12].

SDF can impair fertility for couples using ART. Mechanisms generating this defect in semen include abortive apoptosis, defective maturation and oxidative stress caused by ROS production [1]. Among these mechanisms, SDF occurs frequently during centrifugation as an iatrogenic problem during the handling of samples and exposure to oxygen [5,14]. However, the impact of SDF on clinical outcomes of ART is still unclear, so it is difficult to decide whether testing for SDF should be carried out routinely as part of infertility treatments.

Techniques for determining SDF include the sperm chromatin structure assay (SCSA), terminal deoxynucleotidyl-mediated fluorescein-dUTP nick-end labeling (TUNEL) for apoptosis, in situ nick translation, the single-cell gel electrophoresis assay, acridine orange DNA staining, and the sperm chromatin dispersion test (Halosperm®) [1]. The results obtained by TUNEL and SCSA are occasionally inconsistent [16], so there is no obvious consensus as to which assay method is best correlated with the successful pregnancy rate in ART [1]. Although SCSA is considered to be a precise and repeatable test to detect SDF [17], ART clinics need access to expensive flow cytometry apparatus and specific software for detecting SDF. Therefore, we chose the The Halosperm® to assess SDF in sperm in this study because it can be performed simply using a light microscope. The Halosperm® is a standardized kit for applying sperm chromatin dispersion assays to measure SDF [18].

The general prevalence of SDF varies between studies and detection methods. Belloc et al., surveyed 1974 normozoospermic semen samples and 4345 samples from infertile men, and the cutoff value of >30% SDF was significantly more prevalent in the infertile group [19]. In the normozoospermic group, the mean SDF value was 17.6%. Evenson et al., suggested that an SDF value of 25% increases the time taken to achieve a natural pregnancy, reduces the odds of pregnancy following intrauterine insemination, increases the miscarriage rate or can indicate complete male infertility [17]. Avendano assessed the prevalence of DNA fragmentation as TUNEL-positive spermatozoa with a rate of 3.9% in a fertile group of men versus 9.8% in a subfertile group [20].

In the present study, 27.7% of spermatozoa in the initial semen samples showed SDF. This general prevalence of SDF in normo-spermic men is not meaningful, as previous studies separated groups into two according to SDF rates by <10% to >35% to analyze the influence of SDF on pregnancy [21-23]. However, the SDF value of 27.7% in the initial samples of this study is likely to be within the normal range.

CONCLUSION

From this study, as the incidence of SDF was lower when sperm sorting was performed by the microfluidic device than by a conventional swim-up technique, this method is expected to be

able to select good quality spermatozoa for ICSI without further damage caused by additional testing in an ART setting.

ETHICAL APPROVAL

This study was approved by the Research Committee of Hanbusa Women' Clinic and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

INFORMED CONSENT

All patients gave their informed consent prior to their inclusion in the study allowing the use of their data for research purposes.

REFERENCES

- [1] Tamburrino L, Marchiani S, Montoya M, Marino EM, Natalie I, Cambi M, et al. Mechanisms and clinical correlates of sperm DNA damage. *Asian J Androl.* 2012;14:24-31.
- [2] Sakkas D. Novel technologies for selecting the best sperm for in vitro fertilization and intracytoplasmic sperm injection. *Fertil Steril.* 2013;99:1023-29.
- [3] Jayaraman V, Upadhyaya D, Adiga SK. Sperm processing by swim-up and density gradient is effective in elimination of sperm with DNA damage. *J Assist Reprod Genet.* 2012;29:557-63.
- [4] Ricci G, Peticarasi S, Boscolo R, Montico M, Guaschiro S, Presani G. Semen preparation methods and sperm apoptosis: swim-up versus gradient-density centrifugation technique. *Fertil Steril.* 2009;91:632-38.
- [5] Aitken RJ, Clarkson JS. Significance of reactive oxygen species and antioxidants in defining the efficacy of sperm preparation techniques. *J Androl.* 1988;6:367-76.
- [6] Iwasaki A, Gagnon C. Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril.* 1993;57:409-16.
- [7] Sakkas D, Alvarez JG. Sperm DNA fragmentation: Mechanisms of origin, impact on reproductive outcome, and analysis. *Fertil Steril.* 2010;93:1027-36.
- [8] Twigg J, Irvine DS, Houston P, Fulton N, Michael L, Aitken RJ. Iatrogenic DNA damage induced in human spermatozoa during sperm preparation: protective significance of seminal plasma. *Mol Hum Reprod.* 1998;4:439-45.
- [9] Zhang X, Khimji I, Gurkan UA, Safaee H, Catalano PN, Keles HO, et al. Lensless imaging for simultaneous microfluidic sperm monitoring and sorting. *Lab Chip.* 2011;11:2535-40.
- [10] Schuster TG, Cho B, Takayama S, Smith GD. Isolation of motile spermatozoa from semen samples using microfluidics. *RFM online.* 2003;7:75-81.
- [11] Matsuura K, Takeanmi M, Kuroda Y, Naruse K. Development and performance of plastic microfluidic sperm sorter. *Fertil Steril.* 2008;90:s241.
- [12] Cho BS, Schuster TG, Zhu X, Chang D, Smith GD, Takayama S. Passively driven integrated microfluidic system for separation of motile sperm. *Anal Chem.* 2003;75:1671-5.
- [13] WHO. Assessment of sperm motility. In Laboratory manual for the examination of human semen and the sperm-cervical mucus interaction 4th ed., 1999. pp. 8-13.
- [14] Alvarez JG, Lasso JL, Blasco L, Nunez RC, Heyner S, Caballero PP, et al. Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile lifetime. *Hum Reprod.* 1993;8: 1087-92.
- [15] Muratori M, Maggi M, Spinelli S, Filimberti E, Forti G, Baldi E. Spontaneous DNA fragmentation in swim-up selected human spermatozoa during long term incubation. *J Androl.* 2003; 253-62.
- [16] Li Z, Wang L, Gai J, Huang H. Correlation of sperm DNA damage with IVF and ICSI outcomes: a systematic review and meta analysis. *J Assist Reprod Genet.* 2006;23:367-76.
- [17] Evenson DP. Sperm chromatin structure assay (SCSA). *Methods Mol Biol.* 2013;927:147-64.
- [18] Evenson DP, Wixon R. Comparison of the Halosperm kit with the sperm chromatin structure assay (ACSA) infertility test in relation to patient diagnosis and prognosis. *Fertil Steril.* 2005;84:846-49.
- [19] Belloc S, Benkhaifa M, Cohen-Bacrie M, Delleac A, Amar E, Zini A. Sperm deovynicleic acid damage in normozoospermic men is related to age and sperm progressive motility. *Fertil Steril.* 2014;101:1588-93.
- [20] Avendano C, Franchi A, Taylor S, Morshedi M, Bpcca S, Oehninger S. Fragmentation of DNA in morphologically normal human spermatozoa. *Fertil Steril.* 2003;91:1077-84.
- [21] Borini A, Tarozzi N, Bizzaro D, Bonu MA, Fava L, Flamigni C, et al. Sperm DNA fragmentation: paternal effect on early post-implantation embryo development in ART. *Hum Reprod.* 2006;21:2876-81.
- [22] Frydman N, Prisant N, Hesters L, Frydman R, Tachdjian G, Cohen-Bacrie P, et al. Adequate ovarian follicular status does not prevent the decrease in pregnancy rates associated with high sperm DNA fragmentation. *Fertil Steril.* 2008;89:92-97.
- [23] Benchaib M, Braun V, Lornage J, Hadj S, Salle B, Lejeune H, et al. Sperm DNA fragmentation decreases the pregnancy rate in an assisted reproductive technique. *Hum Reprod.* 2003;18:1023-28.

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