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ORIGINAL ARTICLE

Sperm Biology

Implementation of an in-house flow cytometric analysis of DNA fragmentation in spermatozoa

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An increased amount of DNA fragmentation in the spermatozoa (SDF) is linked to male infertility. The Sperm Chromatin Structure Assay (SCSA) is widely used for analysis of SDF. However, the current software (SCSASoft®) linked to this assay is licensed and often located within larger diagnostic centers. In this study, we present a protocol for using other types of software than SCSASoft® to determine the SDF index (DFI) with clinical relevance. This protocol is engineered after collecting and analyzing 254 samples from fertility patients and sperm donors over a 15-month period. DFI is analyzed using a strict protocol where the spermatozoa are treated with a strong acid (pH 1.2) followed by acridine orange. DFI is determined by a standard flow cytometric software, FACSDiva 6.1.3. Analysis of the outcome of the fertility treatment is included for 137 patients receiving either intrauterine inseminations (IUI) or timed coitus (TC). The results show that the chance of pregnancy declines as DFI increases. We also found that the male DFI affects the chance of pregnancy independent of the female age. We have shown that a standard flow cytometric software can be used when determining a clinical relevant DFI. These findings are a significant step toward implementing the analysis as a part of the routine, in-house diagnosing of the male fertility patient and subsequently optimizing the treatment course of the couple with reduced human and financial costs.

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INTRODUCTION

It is estimated that about 50% of all cases of infertility involve a male factor, either exclusively or in combination with a female factor. Even though the fertility patients are subjected to a careful diagnosing, 15% of the couples are left with a diagnosis of idiopathic infertility.[1,2] One underlying reason for the substantial amount of fertility patients not receiving a proper diagnosis can be found in the analysis of the sperm parameters. The semen sample and spermatozoa are often evaluated by the classical semen parameters such as volume, number of spermatozoa, motility, and morphology. However, this analysis is not always sufficient to estimate the fertility potential of the spermatozoa.[3] There is an increasing focus on the paternal contribution to the fertilization and subsequently how the internal quality, determined by the amount of DNA fragmentation in the spermatozoa, affects the fertility potential.[4] The connection between increased amount of DNA fragmentation in the spermatozoa and subfertility has been described numerous times and has a long history.[5] Comparison studies of fertile and infertile males have shown that the amount of DNA damage and abnormal chromatin packing are significantly higher in the latter group, even when the patients present with normozoospermia.[6–10] It is estimated that up to 20% of male fertility patients with semen parameters otherwise suitable for intrauterine insemination (IUI) treatment present with an increased amount of DNA fragmentation, resulting in pregnancy rates as low as 3%–4%.[6,11–16] If not indicated otherwise, IUI is often the first line of treatment for infertility, which means that every fifth infertile couple could receive fertility treatment with a very poor chance of success.

Recent studies have shown that pregnancy rates after conventional *in vitro* fertilization (IVF) might also be affected by an increased amount of DNA fragmentation, but that fertilization rates after intracytoplasmic sperm injection (ICSI) seem unaffected.[17] However, even though the fertility rates seem unaffected, there is an increased risk of missed abortion if the male has an increased amount of DNA fragmentation.[18–22] Determination and handling of DNA fragmentation is thus still relevant even though the couple receives IVF or ICSI treatment.

There are several methods for measuring the DNA fragmentation index (DFI) in spermatozoa.[14,23] The most widely used methods to evaluate the amount of DNA fragmentation are terminal deoxynucleotidyl transferase-mediated fluorescein-dUTP nick-end labeling (TUNEL), the sperm chromatin dispersion (SCD) test, the alkaline comet assay, and the sperm chromatin structure assay (SCSA).[24,25] However, it has been difficult to obtain interlaboratory standards, and diverse results in the best method concerning predictability of pregnancy have been seen.[24,26] The two flow cytometric assays (TUNEL and SCSA) have the highest reliability and reproducibility for determining DFI, and comparison studies have shown an association between the results obtained from these two

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methods.[25,27,28] However, it is of crucial importance that the analysis is applicable in the daily work in the fertility clinic. The SCSA protocol has a clear advantage: it is possible to freeze samples at −80°C making it possible to collect numerous samples before analysis, and the specific protocol is relatively easy to implement and is not time-consuming.[29,30] However, in 2005, the SCSA test was commercialized and centralized to larger diagnostic centers with a license from the SCSA group. Only two of these were located in Europe. Fertility clinics were encouraged to ship samples to these laboratories to have the analysis done. Within few days, data would be returned to the clinic containing a value for DFI and for the proportion of spermatozoa with abnormal chromatin packaging, categorized as high DNA staining (HDS).[31] This commercialization of the analysis has resulted in a significant increase in the costs associated with the analysis. If the aim is to screen all fertility patients consulting the clinic, the costs will be insurmountable.

The aim of the current study is to describe the implementation of a variant of the sperm chromatin structure assay using standard flow cytometric software (FC-DFI) making it possible to implement the assay in the routine, in-house, diagnostic evaluation of the male fertility patients. Subsequently, we wished to confirm the association between pregnancy rates after IUI or timed coitus (TC) and FC-DFI. Finally, we calculated the pregnancy rate after FC-DFI correlated by the female age.

Combined with the development of smaller and cheaper benchtop flow cytometers, the possibility of using standard flow cytometric software is a significant step toward implementing this analysis within the fertility clinics. This will increase the diagnosing of the male fertility patients and subsequent result in a better prediction for pregnancy outcome.

PARTICIPANTS AND METHODS

Ethical approval

The study is approved by the Danish Data Protection Agency (Journal number 2014-41-3661; Copenhagen, Denmark). Informed consent from all participants was obtained before collection. The committee for health research ethics in Denmark has been contacted. They have confirmed that the study does not need approval from this authority.

Study participants and location for collection

Ejaculated sperm samples were collected over a 15-month period (October 2014 to December 2015) from fertility patients at a private fertility clinic, AAGAARD Fertility Clinic in Skejby, Denmark, and from sperm donors in a private sperm bank, Skejby CryoBank, Denmark.

Inclusion and exclusion criteria

Inclusion criteria were male patients or donors over 18 years old. Sperm samples with $<3 \times 10^6$ spermatozoa per ml were excluded from the study owing to the technical specification of the analysis.

Collection of sperm samples

The fresh sperm samples were analyzed in accordance with World Health Organization guidelines 5th edition within 1 h from delivery. Two aliquots of 100 µl raw semen were collected in a 1.5 ml micro tube (easy cap, Sarstedt, North Rhine-Westphalia, Nümbrecht, Germany) and flash frozen at −80°C and kept there until analysis for DNA fragmentation was performed.

Principles for the DNA fragmentation assay

The flow cytometric DNA fragmentation assay utilizes the cell permeant reagent acridine orange (AO), which interacts with double-stranded as well as single-stranded DNA. When intercalated to double-stranded DNA (dsDNA), AO has an excitation maximum at 502 nm and an emission maximum at 525 nm (green). When bound to single stranded DNA (ssDNA), the excitation maximum of AO is 460 nm and the emission maximum is 650 nm (red). Therefore, a flow cytometer with a 488 nm blue laser can be used to measure the ratio of dsDNA versus ssDNA. To detect the amount of dsDNA versus ssDNA, the raw sperm sample was initially treated with a strong acid (pH 1.2) for 30 s.

Specimen collection

An aliquot of 100 µl of raw semen was collected and frozen at −80°C during the primary evaluation of the patients' sperm samples. The patients had been instructed on 2–3 days of abstinence. The analysis was performed with regularly intervals when convenient.

Reagents and utensils

The reagents needed are an acid solution (pH 1.2), a coloring buffer, a TNE buffer and a 0.0015% acridine orange staining solution. The specifications for the reagents, solutions, and utensils are shown in **Supplementary Information** (**Appendix 1**).

Reference sample

Approximately one reference per 10 samples analyzed is preferential. It should be from a donor and contain spermatozoa with both intact and damaged DNA. The reference sample can with advantage be shared with another laboratory performing the same analysis in order to detect any fluctuations in the procedure.

Sperm preparation

On the day of the analysis, samples were thawed on ice and diluted in 5 ml tubes to a concentration of 6×10^6 ml^{-1} in a total volume of 200 µl according to **Table 1**. All samples are diluted at least 1:1. Samples with a concentration between 3×10^6 ml^{-1} and 12×10^6 ml^{-1} will thus have a lower concentration than 6×10^6 ml^{-1}. However, data must still be collected for 10 000 cells. Samples were kept in a rack standing on ice (from the −20°C freezer). Four hundred microliters of acid solution was added to the samples. After exactly 30 s, 1.2 ml acridine orange was added. Samples were left on ice to equilibrate in the dark for 3 min.

Flow cytometric analysis of DNA fragmentation

Cells were analyzed by a FACSCanto™ II flow cytometer (Becton, Dickinson and Company [BD], NJ, Franklin Lakes, USA) with a blue

Table 1: Dilution factor

Dilution factor	*Sample volume (µl)*	*TNE* buffer (µl)*
1:2	100	100
1:3	67	133
1:4	50	150
1:5	40	160
1:6	33	167
1:8	25	175
1:10	20	180
1:15	13	187
1:20	10	190
1:25	8	192
1:30	6	194

Samples are diluted in 5 ml falcon tubes, labeled with patient ID, to a concentration of 6×10^6 ml^{-1} in a total volume of 200 µl. Division of the actual concentration with 6 will provide the dilution factor. By following the above table, the proper concentration in a 200-µl volume is assured. All samples will as a minimum be diluted in a 1:1 ratio. If samples contain a concentration between 3×10^6 and 12×10^6 spermatozoa per ml, a final concentration below 6×10^6 ml^{-1} is obtained. The sample will thus need to be analyzed through the flow cytometer for a longer period of time in order to obtain 10 000 cell counts. *TNE: Tris-HCl NaCl EDTA

laser (488-nm, air-cooled, 20-mW solid state). Ten thousand events were collected from each measurement. The flow cytometric dot plot was analyzed with FACSDiva 6.1.3 (BD). This is standard flow cytometric analysis software supplied from BD alongside the flow cytometer. A pilot study has shown that the flow cytometric software FACSDiva 6.1.3 results in a DFI in an agreement to a DFI obtained by SCSASoft, the original software used for this assay. In the initial implementation of the assay, a flow cytometric template can with advantage be created within the FACSDiva 6.1.3 software. For example of setting up template, see **Figure 1** and **Supplementary Information** (**Appendix 2**).

Sample analysis

A prefabricated template was retrieved or a new sheet is created. Samples were placed in the sample injection tube (SIT) and "Aquire" was pressed. As the SIT holds 30s dead volume, it is recommended to run samples for approximately 15 s before recording is initiated. Optimal analysis is obtained after 10 000 events has been recorded.

Determination of DFI using FACSDiva 6.1.3

Even though a template had been used, a manual inspection of the gates is always advisable due to slight variance in the localization of the cell populations within samples. DFI is calculated as the percent-wise amount of damaged DNA from the total (**Figure 1**). For quality control and regular maintenance, see **Supplementary information** (**Appendix 3**).

Analysis of pregnancy rate

Pregnancy rates are calculated from a subpopulation of the patients receiving fertility treatment by IUI (stimulated or nonstimulated) within a 6-month period from the DFI analysis. Furthermore, pregnancies as a result of TC or SP occurred during the treatment period were also registered. Pregnancies were all confirmed by an ultrasound in gestation week 6 or 7. Pregnancy rates were calculated per cycle. The classical thresholds for DFI are between 0 and 19.99 for normal DFI, moderate DFI is between 20 and 29.99, and increased DFI ≥30. We added two additional divisions. The group with low amount of DNA fragmentation (0–19.99) was divided into 0–9.99 and 10–19.99 to investigate a possible difference within this rather large range. To further clarify the impact of moderate DFI on pregnancy rates, this group was subdivided into two groups: DFI between 20 and 24.99 and DFI between 25 and 29.99.

As DFI increases with the male age, it is important to investigate whether a possible decrease in pregnancy rates in couples with increased DFI is a matter of increasing female age. Patients were divided into four groups: Group 1, low DFI, low female age (DFI <20, age ≤30 years); Group 2, low DFI, high female age (DFI <20, age >30 years); Group 3, moderate/high DFI, low female age (DFI ≥20, age ≤30 years); and Group 4: moderate/high DFI, high female age (DFI ≥20, age >30 years). Pregnancy rates were calculated for each group. Significance of the difference between groups was determined by a Chi-squared test.

RESULTS

A total of 272 sperm samples were collected from 147 fertility patients and from 34 sperm donors. Eighteen samples did not meet the inclusion criteria of a total concentration of ≥3 × 10^6 spermatozoa per ml leaving 254 samples for analysis. To determine pregnancy rates, data on

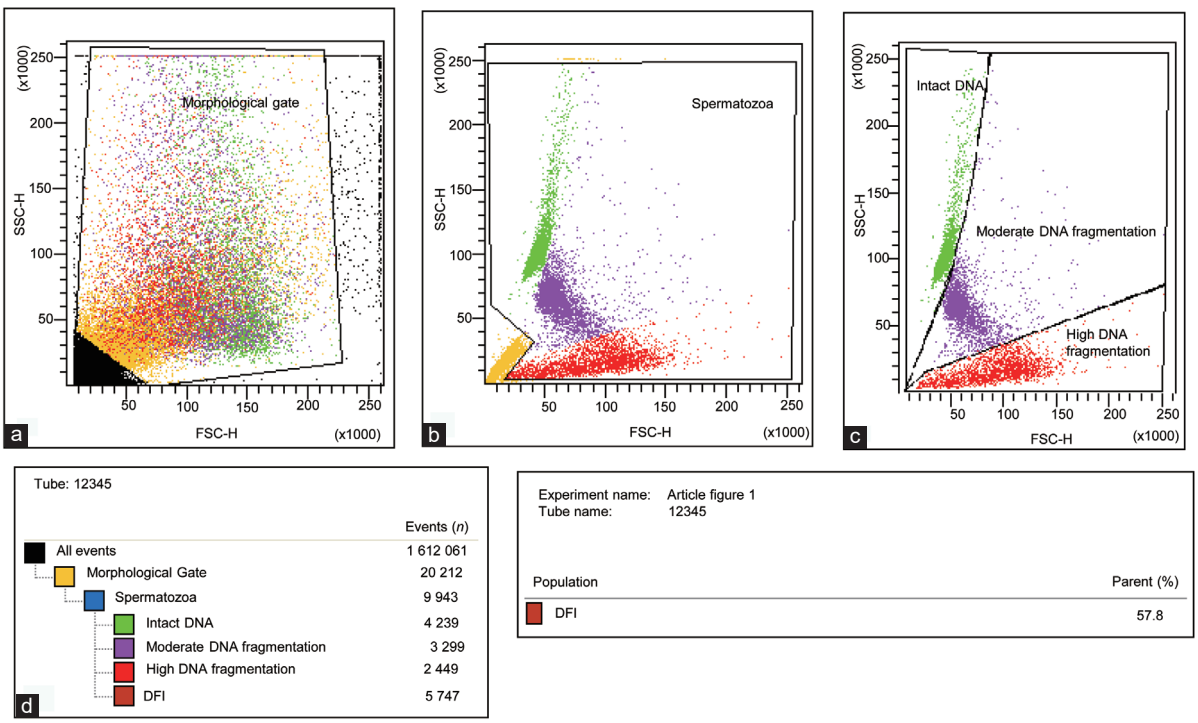

Figure 1: Flow cytometric dot plot. (**a**) The classical "flame-shaped" cluster of spermatozoa in the flow cytometric output. The spermatozoa can be selected by applying a "morphological gate." (**b**) The cells from the morphological gate are shown in a new window distributed by the red and green fluorescence. The setting of the gate makes it possible to exclude debris or any outliers. (**c**) The gated cells from **b**. This window allows for gating in subpopulation. The green population is spermatozoa containing intact DNA. The purple and red population contains spermatozoa with various degree of DNA fragmentation. The blue cells illustrate spermatozoa containing a tight chromatin packing. (**d**) A quantification of the dot plot. It is now possible to obtain the FC-DFI by calculating the percentage of fragmented spermatozoa in relation to the total number of spermatozoa. SSC-H: side scatter-heigth; FSC-H: forward scatter-height; FC-DFI: flow cytometric DNA fragmentation index.

pregnancy were obtained from the subgroup of 137 patients receiving IUI treatment. A total of 310 cycles were investigated.

FC-DFI

Pregnancy rates for the first two groups (DFI: 0–9.99 and 10–19.99) were 28.1% and 26.5%, respectively. If DFI was ≥20, pregnancy rates decreased to 10%. This is a significant decrease in pregnancy rates compared with the group with a DFI between 0–19.99 (P = 0.003, t-test). If DFI was ≥30, the pregnancy rates dropped to 5%. The difference in pregnancy rates between the group with normal FC-DFI (0–19.99) and the group with severely increased FC-DFI (≥30) was statistically significant (P = 0.03, t-test). Twenty-five of the 137 patients receiving fertility treatment (18.2%) presented with a FC-DFI above 20. Almost 10% of the patients had a FC-DFI of 25 or more and 4.4% of the patients had a FC-DFI of 30 or more. These results are shown in **Table 2** and illustrated in **Figure 2**.

The current results as well as the literature show that a low pregnancy rate can be expected when DFI increases to 30 or more. As DFI increases with the male age, we considered whether the decrease in pregnancy rates was caused by an increasing female age. The couples were divided into four groups and the pregnancy rate per cycle was calculated. Group 1 had a pregnancy rate per cycle of 30.8%, Group 2 of 23.3%, Group 3 of 8.3%, and Group 4 of 12.1%. A Chi-squared test showed that the pregnancy rates were dependent on DFI (P = 0.01, t-test). To detect in which group the dependence was strongest, the Chi-squared test was performed on the various groups. The difference in pregnancy rate between Group 1 and Group 3 was significant (P = 0.006, t-test).

Variability

The variability has been checked by a reference sample. This sample has been collected one time (one ejaculation), frozen in aliquots, and analyzed 73 times on different days and personnel. These data show a mean value of 9.1 with a coefficient of variance of 0.0993. This shows low variance of the method.

DISCUSSION

We have demonstrated that it is possible to implement the DFI analysis as a standard, in-house diagnostic tool for the male fertility patients using a strict protocol and standard flow cytometric software. The subsequent DFI results reveal that pregnancy rates decline as FC-DFI increases. When FC-DFI is ≥30, the pregnancy rate is only 5% per cycle. These results are in tune with the literature for other DNA fragmentation analyses. However, the number of couples included in each group does not allow us to state significant finding. It has previously been observed that a moderate DFI might also affect the pregnancy rates.[16] In the current study, we also found that the chance of pregnancy per cycle started to decrease when DFI was increased above 20.

We also investigated whether the decrease in pregnancy rate is merely due to increasing female age. A decrease in pregnancy rates was seen between the group with low female age and low DFI (DFI <20, age ≤30 years) and the group with low female age and increased male DFI (DFI ≥20, age ≤30 years). Even though the females were all 30 years old or younger, the pregnancy rates dropped from 30.8% to 8.3% if their male partner had a DFI of more than 20. This shows that the chance of pregnancy is linked to the male partner's DFI. No significance was found among the rest of the groups. The above results show that a DFI obtained by standard flow cytometric software can be used as an in-house diagnostic tool with clinical relevance.

However, it is encouraged that patients presenting with an increased DFI (above 30%) have a second semen sample analyzed in order to confirm the result. It is estimated that up to 15% of patients may shift from increased DFI to low or vice versa in a secondary analysis.[16] However, it is believed that DFI result within an individual is more consistent than the classical semen parameters.[11]

Strengths and limitations

The strength of the study is that the samples have been collected from a heterogeneous group containing both fertility patients receiving treatment and donors with a proven fertility. This ensures that multiple ranges of DFI have been evaluated. In FACSDiva, DFI is determined from an area plot that allows the detection of multiple dots located on top of each other. This results in a three-dimensional gating, which gives a clear distinction between cell populations and ease the setting of gates, resulting in a more precise determination of DFI compared to the histogram often used.

There are some limitations to the study. The study population for pregnancy rates in the various FC-DFI groups was small and the study should be examined in a larger set up. Furthermore, it was not possible to collect data on possible confounders such as diet, exercise, smoking, and BMI.

Pregnancy rates were calculated from the time of DFI measurement and 6 months. A few fertility patients had received treatment before their DFI analysis. This might have resulted in a slightly higher pregnancy rate, as some negative cycles might not have been registered.

The aim of the study was to show that the analysis for DNA fragmentation could be established with in-house equipment and a

Table 2: Pregnancy rates

FC-DFI	*No. patients*	*No. cycles*	*No. pregnancy*	*Pregnancy rate/ per cycle (%)*	*95% CI*
0–9.99	43	89	25	28.1	19.1–38.6
10–19.99	69	151	40	26.5	19.6–34.3
20–24.99	12	29	4	13.8 (P=0.12)	3.9–31.7
25–29.99	7	21	2	9.5 (P=0.08)	1.2–30.4
≥30	6	20	1	5 (P=0.03*)	0.1–24.9
Total	137	310	72		
≥20[a]	25[a]	70[a]	7[a]	10[a] (P=0.003*)	4.1–19.5[a]

Patients have been divided into groups where the male has a FC-DFI between 0–9.99, 10–19.99, 20–24.99, 25–29.9, and ≥30. Pregnancy rates per cycle have been calculated. The P values are displayed in the brackets. All P values have been calculated with DFI <20 as control group. The results for FC-DFI are displayed in **Figure 2.** Standard deviation is 10.28%. FC-DFI: flow cytometric DNA fragmentation Index. [a]Pregnancy patients divided. *P<0.05 for t-test. CI: confidence interval

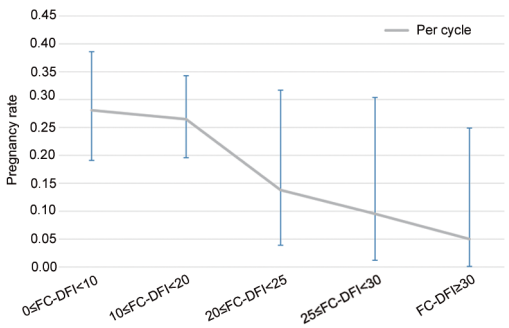

Figure 2: Graphical illustration of pregnancy rates. Pregnancy rates per cycle after IUI drop from 28.1% if the FC-DFI is below 10% to 5% if FC-DFI is 30 or more. Trend lines illustrate the 95% confidence interval. FC-DFI: flow cytometric DNA fragmentation index; IUI: intrauterine insemination.

standard flow cytometric software. This aim could be extended by including other types of flow cytometers and software.

Perspective

The results above reveal that the pregnancy rates are already affected when FC-DFI exceeds 20. More than 18% of patients receiving IUI treatment in this study present with FC-DFI at or above 20. This means that almost every fifth couple otherwise suitable for IUI has a reduced chance of pregnancy after IUI. It, thus, seems highly relevant that this parameter be included in the routine diagnosis of the male patient. At the moment, the only treatment believed to circumvent increased DNA fragmentation is ICSI, possibly after TESA. This can seem as an invasive treatment for couples with normozoospermia or mild oligozoospermia. However, the chances of success after IUI are small. By including the DFI analysis in the diagnosis, it will be possible to spare these couples from multiple unsuccessful IUI treatments. However, in the end, the true value of this analysis is obtained when we have mapped possible therapeutic interventions for these patients. Preliminary studies point to antioxidants/lifestyle changes or shorter abstinence time to improve DFI in patients with increased amount of DNA fragmentation.[32,33] However, further studies are needed in these areas.

If it is possible to reduce an increased DFI in a male fertility patient, the couple might end up with an ongoing pregnancy after IUI or even by natural conception. This will decrease the number of patients needing to be referred to the more invasive IVF or ICSI treatment. Thereby reducing the costs, both financial and human, inevitably connected to fertility treatment. Furthermore, an "in house" analysis will significantly reduce the costs previously connected to this analysis when it was performed in larger commercial centers.

CONCLUSION

We have demonstrated that DFI can be determined with in-house equipment and standard flow cytometric software other than the one currently linked to this assay (SCSASoft®). Furthermore, this assay can establish a clinical relevant DFI. The results on pregnancy rates of this study are in tune with the current literature in the field for flow cytometric determination of DNA fragmentation in spermatozoa. When taken into account that the development of smaller and cheaper benchtop flow cytometers continues, these findings underline the fact that this analysis is ready to be fully implemented as a routine, in-house diagnostic tool of the male fertility patient.

AUTHOR CONTRIBUTIONS

ASR, JA, and JF have all contributed to ideas, data collection and analysis, and completion of article. CW has contributed with statistical analysis. All authors read and approved the final version of the manuscript.

COMPETING INTERESTS

All authors declared no competing interests.

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Supplementary Information is linked to the online version of the paper on the *Asian Journal of Andrology* website.

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SUPPLEMENTARY INFORMATION

Appendix 1

Specifications and recipes of the reagents, solutions, and utensils.

Acid solution (ordered from the local pharmacy)

- 20 ml 2.0 M HCl (0.08 M)
- 4.39 g NaCl (0.15 M)
- 0.5 ml Triton X-100 (1%)
- ddH_2O to 500 ml
- Adjust to pH to 1.2 med 5 M HCl.

Coloring buffer pH 6.0 (Ordered from the local pharmacy)

- 370 ml 0.1 M Citric acid
- 630 ml 0.2 M Na_2PO_4 buffer
- 372 mg EDTA (disodium, F.W. = 372.24 1 mM)
- 8.77 g NaCl (0.15 M).

Mix overnight in order for the EDTA to dissolve. Adjust pH to 6.0.

TNE buffer 10X, pH 7.4 (ordered from the local pharmacy)

- 9.48 g Tris-HCl (158 0.1 M)
- 52.6 g NaCl (F.W. 58.44 1,5 M)
- 2.23 g EDTA (disodium, F.W. = 372.24 10 mM)
- ddH_2O to 600 ml
- Adjust to pH 7.4 using 2 M NaOH.

Before use, the 10 × TNE buffer is diluted to 1 × TNE buffer. Add *e.g.*, 10 ml 10 × TNE to 90 ml ddH_2O.

Storage time up to 1 year at 5°C.

Acridine orange staining solution (0.015%) from Polyscience, 400 Valley Road, Warrington.

A 0.015% AO solution can *e.g.*, be obtained by mixing 6 µl of the AO stock solution 2% in 20 ml coloring buffer. The solution must be protected against sunlight.

Storage time up to 2 weeks at 5 °C.

Utensils:

- FACSCanto II (Becton, Dickinson and Company, (BD), New Jersey, USA)
- Falcon tubes 5 ml (Falcon, Reynosa, Mexico)
- Multi pipette (Eppendorf, Hamburg, Germany)
- 10 and 25 ml Combitips Advanced (Eppendorf, Hamburg, Germany)
- Pipette – 10 µl, 200 µl, 1000 µl (ThermoScientific, Waltham, Massachusetts, USA).

Notes:

Samples are to be kept in a rack standing on ice (from the −20°C freezer). If the conditions are too cold, the effect of the acid on the samples will be impaired.

It is advised to keep the flow rate of the flow cytometer on "Low" and an event per second on app. 500. However, the paramount issue is to have a clear distinction of the different cell population. A high flow rate is not advisable as the alignment of the cells might be compromised.

Appendix 2

This template can be retrieved with every analysis in order to save time and ensure uniformity in the assay.

Creation of template in FACSDiva 6.1.3.

- Open FACSDiva 6.1.3 on the computer
- A template is created where the Windows "Browser," "Cytometer," "Worksheet" and "Acqusition Dashboard" is visible. The worksheet is initiated in global worksheet and 3 minor dot plot is created with FSC-H on the X-axis and SSC-H on the Y-axis
- This laboratory currently has the photomultiplier tubes (PMT) voltage located approximately around 485 for the FSC parameter, 515 for the SSC, 310 for the green parameter, and 445 for the red parameter. These values may vary slightly between different flow cytometers and may also need slight adjustments after new baseline, new beads or sometimes after a long clean
- In global worksheet, three minor dot plots and two statistics views are created
- In the first dot plot the spermatozoa and other types of cells located in this area are shown
- A polygon gate is set around the characteristic flame-shaped cluster of spermatozoa, **Figure 1a**. This ensures that the debris is left out without excluding any outlying spermatozoa. We call this the "morphological gate"
- The next dot plot is created so the population from the morphological gate is shown. The X-axis shows Red-H and the Y-axis the Green-H. A characteristic distribution of the spermatozoa is seen in the dot plot, **Figure 1b**

- The spermatozoa are gated by a polygon gate in such a way that the debris in the lower left corner and alongside the edges is excluded. This gate is named "Total Spermatozoa"
- The next dot plot has the same axis as the previous; the cell cluster is however only from the gate "total spermatozoa"
- The cell cluster is divided into three distinct populations by setting gates for the normal spermatozoa and for the moderate and highly fragmented spermatozoa
  - The population seen to the left with an upward distribution are cells containing no or minimal amount of DNA fragmentation
  - The cells seen in a 45° angle to the lower right are spermatozoa containing a moderate amount of DNA fragmentation
  - The lower cluster of cells with a horizontal orientation is cells containing a high amount of DNA fragmentation. **Figure 1c** shows the localization of the three distinct gates.
- From this window, a population hierarchy is added showing total events for each gate and the percent of each population compared to the "parent" cluster. As DFI is the amount of DNA fragmentation compared to the total population of cells 31, it is possible to determine DFI merely by adding the amount of moderate highly fragmented spermatozoa and calculating the percentwise amount of the total, **Figure 1d**
- The template can now be saved and retrieved for every analysis.

Appendix 3

Quality control and regular maintenance.

In order to maintain optimal instrument performance, a quality analysis was performed with every start-up of the flow cytometer. This was done to evaluate the current flow cytometer performance toward a previously established baseline. After warming of the lasers and the fluidic startup was completed, the cytometer setup and tracking interface (CST) mode was entered. BD™ Cytometer Setup and Tracking Beads (CSTB) used for the quality control are designed for quality control of several of the BD flow cytometers and the FacsDiva software version 6.0 or later. The CSTB reagent consists of three equal concentrations of polystyrene beads of a dim, midrange, or bright intensity with dyes spanning a wide range of both excitation and emission wavelengths. In the quality control, the robust coefficient (CV) of each type of beads and median fluorescence intensity are measured in each fluorescence detector. The use of CSTB ensures a reproducible day-to-day flow cytometer performance and thereby a stable data collection between analysis.

After each time the flow cytometer had been used, a cleaning process was performed. 5-ml tubes were prepared with clean, rinse and ddH_2O solution provided by the flow cytometer manufacturer. The flow cell is cleaned twice and is subsequently set to run for 10 min. Afterward, it is rinsed and ddH_2O is run through the hoses. Shutdown and regular maintenance is performed. Extensive cleaning is necessary when working with acridine orange, as it tends to "stick" to the hoses.