Flow Cytometry to Assess Sperm DFI and HDS Quality in Order to Assess the Best Washing Method in Oligoasthenospermic Patients.

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Abstract:

Sperm is more than just a bearer of paternal genetic information; it has a purpose much beyond fertilization. The integrity of the sperm genome is a must for the birth of healthy kids, and sperm screening should include DNA integrity testing. DNA integrity analysis is a more accurate diagnostic and predictive indicator of sperm fertility. The DNA fragmentation rate is now one of the most widely used indicators for assessing sperm fertility and predicting pregnancy outcomes. Oligospermia is a condition in which a man's sperm count is abnormally low. The condition is known as Oligozoospermia when the sperm concentration is fewer than 15 million per milliliter. This research employs Flow Cytometry (FC) to assess sperm DFI (DNA fragmentation index) quality in order to assess the best washing method in Oligoasthenospermic patients using FC Sperm Chromatin Structure Analysis (SCSA). The SCSA test is most commonly used to evaluate the percentage of sperm with fragmented DNA. Due to a lack of full protamination and consequently an elevated quantity of retained histones, the High DNA Stainable (HDS) sperm population in a semen sample has an abnormally high level of DNA staining. The different washing procedures of Swim up (SU), Density Gradient (DG), and Magnetic activated cell sorting (M) method were used and it was found that the Density Gradient Method showed low DNA fragmentation index and is suitable for Oligoasthenospermic patients for Assisted Reproductive Technology (ART) procedures.

Keywords: Semen processing, Swim up, Density gradient, Magnetic Activated cell sorting.

Introduction

Sperm DNA integrity is a critical sperm quality measure in the prognosis of infertility and the success of assisted reproductive techniques. The assessment of sperm quality in a basic andrology laboratory is based on World Health Organization [1, 2] (WHO) criteria, which are poor predictors of reproductive outcome. Sperm factors such as concentration, motility, and morphology are emphasized in sperm analysis, according to WHO guidelines. Traditional

analysis establishes both qualitative and quantitative threshold values for the above-mentioned characteristics. Although fertile men have greater mean sperm parameters (concentration, motility, and morphology) than infertile men as a group, fertile and infertile men have significant overlap [3]. A normal spermiogram is found in about 15% of infertile men [4]. Recent research has underlined the importance of sperm DNA integrity as a key determinant influencing sperm functional competency [5]. To quantify sperm DNA damage, various techniques have been developed and deployed in research laboratories, which are more therapeutically useful and relevant. However, only a few andrology facilities have included DNA integrity testing in their standard semen analysis. Because these modern assisted conception techniques bypass the normal selection barriers of conception, sperm with substantial DNA damage may increase the risk of passing genetic aberrations to the conceptus, affecting fetal and postnatal development [6].

Oligospermia is a condition in which a man's sperm count is abnormally low. Other characteristics of males with this condition's sexual health are standard. This involves the ability to obtain and keep an erection, as well as the ability to produce ejaculation during orgasm. The number of sperm in your ejaculate can change over time. For fertility, a sufficient amount of healthy sperm is frequently required. Sperm counts of 15 million sperm per milliliter (mL) of sperm are considered usual by the World Health Organization (WHO). Anything below that is deemed low, and Oligospermia is diagnosed [7]. Mild Oligospermia is defined as sperm counts of 10 to 15 million per ml,5 to 10 million sperm/ml is termed moderate Oligospermia. When sperm counts, 0 to 5 million sperm/ml, severe Oligospermia is identified. How many guys have low sperm counts in their sperm is unknown. This is, in part, because not everyone with the illness is diagnosed. Only males who are having trouble conceiving naturally and seek help will be diagnosed.

The sperm chromatin structure assay (SCSA) is a new diagnostic tool for detecting sperm samples with a lot of DNA fragmentation (small breaks in the sperm chromosomes). The assay, which was first published by Even son in 1980, is a flow cytometry test that evaluates sperm DNA's vulnerability to acid-induced denaturation DNA in situ. [8] SCSA calculates the degree of sperm DNA fragmentation due to intrinsic and extrinsic causes and provides it as a DNA Fragmentation Index (DFI). SCSA is used to assess male infertility and subfertility, as well as toxicological research and the quality of laboratory semen samples. DNA fragmented sperm samples with a high percentage of fragmented DNA (>30%) have been linked to a nearly four-fold reduction in term births [9]. It's also linked to multiple miscarriages. Even though a male's sperm count, motility, and morphology are all normal, he may have a significant fragmentation level. It's possible that this has something to do with a couple's infertility issues. Depending on the patient's reproductive history, the SCSA may be recommended. This test does not assess the capacity of sperm to fertilize an egg.

SCSA is a widespread diagnostic tool in the detection of sperm samples with a high degree of DNA fragmentation and absence of histone-to-protamine proteins exchange

in sperm nuclei. [10] Sperm abnormalities are defined by SCSA as higher sperm DNA sensitivity to in-situ heat/acid-induced denaturation. [11] A fully grown and healthy sperm nucleus with a high disulfide bond (S-S) content should theoretically have its DNA retained in double-stranded form. [12] A low pH treatment allows defective sperm DNA to be exposed to the damaged sites. AO molecules are intercalated into double-stranded DNA in intact sperms by acridine orange (AO) staining, whereas AO molecules aggregate at single-stranded DNA in faulty sperms. [11, 12] When flow cytometry (blue light) is used, intact and faulty sperms show green (native DNA) and red (damaged DNA) fluorescence, respectively. [8, 11] Signals will be analyzed using software programming to look at sperm DNA fragmentation (SDF) as well as abnormal chromatin structure. The measurement from 10000 individual sperm is analyzed by the SCSA software to determine the percentage of DFI and percentage of HDS. SCSA is made up of a set of flow cytometry protocols and a software tool called SCSAsoft ®. The DNA fragmentation index (DFI) and the High DNA Stainable (HDS) fraction, which describe the percentage of sperm with DNA breaks/protamine faults and immature spermatozoa without full protamination, respectively, are used to make measurements. [13]

DFI is further subdivided into mean DFI (X DFI) and standard deviation DFI, also known as Cells outside the Main Peak (SD DFI). [12] In terms of sperm DNA integrity, the index has been determined to be the most sensitive parameter for fertility determination. Normal DFI indicates that there is no quantifiable value; a moderate DFI sample indicates that sperm morphology is normal, and high DFI fractions showed elongated nuclei and apoptotic indications. The higher DFI, the greater the likelihood of infertility or sub-fecundity. The rate of natural fertility gradually drops when DFI approaches 20 percent; [8] when DFI exceeds 30 percent, the odds ratio for natural or intrauterine insemination (IUI) fertility is dramatically reduced by 8-10 times, implying a near-zero likelihood of pregnancy. [8]

Due to the presence of unprocessed P2 protamine, the HDS sperm population shows a surprisingly high degree of DNA staining by AO molecules. [14, 15] The HDS value is calculated based on structural chromatin anomalies. A high HDS value indicates immature sperm morphology and, as a result, infertility. [15]

The three common methods of DG, SU, and M are to be compared here for the purpose of this research. The swim-up technique is the most popular in IVF labs, and it is chosen if the sperm sample has a normal quantity of healthy sperm (Normozoospermia). Sperms are chosen based on their motility and ability to swim out of the seminal plasma using this method.

If a "direct swim up" (DS) is used, the full volume (well mixed) is separated into fractions of 1 ml and placed in centrifuge tubes after liquefaction (round bottom is preferred). In each tube, 1.3 ml of culture medium is carefully deposited over the sperm. The tubes must be placed in the incubator at a 45° angle and incubated for 30-60 minutes at 37° C. By inclining the tubes at 45 degrees, we increase the surface area between the medium and the sperm, which improves the sperm's ability to swim

out of the sperm and into the medium. After that, place the tube in the vertical position and carefully extract 1 ml of the supernatant from each tube, aspirating the sperms from the upper meniscus downwards with a sterile pipette [16].

In cases of severe Oligozoospermia, Teratozoospermia, or Athenozoospermia, DG is the best method for selecting a larger number of motile spermatozoa. By using a density discontinuous gradient, good quality sperms can be isolated from dead sperms, leukocytes, and other seminal plasma components. The colloidal silica covered with silane of the gradient can be used to select cells with varying density and motility during centrifugation; sperms with high motility and good morphology are at the bottom of the tube, eventually free of dead spermatozoa, leukocytes, bacteria, and debris two-layer density-gradient with a top layer of 40% (v/v) and a lower layer of 80% (v/v) is the most commonly used discontinuous density-gradient. [17].

Magnetic activated cell sorting (M) distinguishes between apoptotic and non-apoptotic spermatozoa. Phosphatidyl serine residues are translocated from the spermatozoa's inner membrane to the outer membrane during apoptosis (programmed cell death). Although Annexin V has a high affinity for Phosphatidyl serine, it is unable to penetrate through the intact sperm membrane. MACS is used to separate dead and apoptotic spermatozoa using colloidal super-paramagnetic beads (50 nm in diameter) linked to highly specific antibodies to Annexin V. Annexin V binding to spermatozoa suggests that the sperm membrane integrity has been disrupted [18].

Semen Cryopreservation: -

- ❖ I st step sperm wash procedure
- ❖ Check for sperm count and motility
- ❖ Keep the freezing medium at room temperature for30 minutes.
- ❖ Slowly add the first few drops (3-4) drops freezing medium to semen and gently mix at room temperature
- ❖ Add an equal volume of freezing medium and mix well.
- ❖ Transfer the above mixture into a sterile vial.
- ❖ Label the cryovials properly.
- ❖ Keep the cryovials in the refrigerator (4^{o}c) for 10 minutes.
- ❖ Freezer (0^{o}c) for 10 minutes LN_2 vapour phase for 1 minute and then plunge in LN_2.

Thawing and processing of cryopreserved semen sample: -

- ✓ Remove the cryovials from the LN_2 container.
- ✓ Thaw the sample at 37^{o}c for 5 minutes.
- ✓ Mix the sample with 1ml volume of sperm wash media.
- ✓ Centrifuge at 1800 rpm for 8 minutes
- ✓ Discard the supernatant
- ✓ Carefully overlay 100 – 200ml IVF wash media (HEPES)
- ✓ Inculcate at 37^{o}c for 1 hour.

Collect the overlay and check for the count at motility

The main aim of this research is to assess sperm DFI quality in order to assess the best washing method in Oligoasthenospermic patients using FC Sperm Chromatin Structure Analysis (SCSA). The following section gives the materials required as well as the method followed.

Materials and Method:

Chemical and Reagents

Acridine orange dye (AO DYE), Sperm wash media (VITROMEDia SAR HEALTH) Sperm freeze media (VITROMEDia SAR HEALTH) Magnetic Activated Cell Sorting (Canfrag), liquid nitrogen, and Flow Cytometry (Beckman Coulter). Semen samples were obtained from 40 Oligoasthenospermic patients with progressive motility <32%, who underwent ART procedures in Sumathi fertility center Madurai between June-october2021.Informed Consent was obtained from all men prior to the study.

Approved by Institutional Review Board (IRB)

This research was approved by the institutional review board of Bharath University (SBDCH/IEC/02/2017/09) Chennai.

Sample Collection

Briefly, the semen sample was subjected to swim up, density, and magnetic method. After washing, the samples were cryopreserved in liquid nitrogen for a period of 3 months. After 3 months DNA fragmentation index and HDS measured by the SCSA method.

Methodology

The method of Flow Cytometry is used in this research. The SCSA can tell the difference between normal sperm and those with DNA fragmentation. This test quantifies the percentage of sperm with intact vs. fragmented chromatin using specific stains, complicated instrumentation (flow cytometry), and a laser beam (DNA).

Protocol: Frozen thaw semen sample treated with low pH buffer (pH 1.20, 30sec) and stained with 1.2ml of Acridine orange dye, measure 10000 sperms by Flow Cytometry.

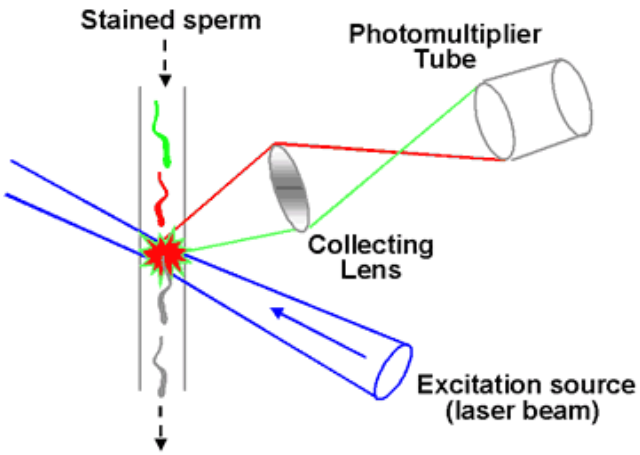

Fig. 1. Flow Cytometry [9]

Flow cytometry is a technique for swiftly analysing single cells or particles that are suspended in a buffered salt-based solution and flow past one or more lasers. Visible light scatters and one or more fluorescence characteristics are assessed for each particle. Visible light scatter is assessed in two directions: forward (Forward Scatter or FSC), which reflects the cell's relative size, and at 90 degrees (Side Scatter or SSC), which indicates the cell's internal complexity or granularity. Fluorescence has no effect on light scatter. Transfection and expression of fluorescent proteins (e.g., Green Fluorescent Protein, GFP), staining with fluorescent dyes (e.g., Propidium Iodide, DNA), or staining with fluorescently attached antibodies are used to prepare samples for fluorescence measurement (e.g., CD3 FITC) [19].

Steps:

The following are the steps involved in this research:

1) Semen sample (n=40) sperm concentration :< 15 million/ml.motility<32%

2) Sperm processing (or) washing procedure

Swim-up

Density gradient method

Magnetic activated cell sorting method

3) Cryopreservation and thawing

Evaluate DFI and HDS by SCSA METHOD.

The DFI & HDS values are calculated for each method of DG, SU, and M, and the results are tabulated in the next section.

Statistical Analysis:

Statistical Analysis was done by using SPSS Version 26. One Way ANOVA and Independent's-test will be used to find the significant difference between groups. P<0.05 will be considered statistically significant.

Results:

DFI is expressed as mean &standard deviation. The results are shown in the following tables.

Table 1:

Sample		Mean	SD	P-Value
DFI	SU	18.25	6.79	0.006 Sig
	DG	8.76	.98	
	M	9.31	2.23	
HDS	DS	.32	.19	0.021 Sig
	DG	.31	.23	
	M	.30	.15	

The above table compares the DFI Type in both group was means differences between the groups. The mean DFI Density Gradient Method mean was 8.76 ± 0.98, Magnetic Activated mean was 9.31 ± 2.23 and Swim up Method mean was 18.25 ± 6.79. Statistically significant (P<0.006). Similarly, HDS Type Swim up Method mean was 0.32 ± 0.19, Magnetic Activated mean was 0.30 ± 0.15 and Density Gradient Method mean was 0.31 ± 0.23. there was no statistically Significant(P>0.05).

Table-2

	Control	Mean	SD	P-Value
DFI	SU	4.53	3.46	0.025 Sig
	DG	4.40	3.71	

	M	12.04	5.55	
HDS	SU	.43	.12	0.002 Sig
	DG	.24	.07	
	M	.15	.10	

The above table compares the DFI Type in both groups as means of differences between the groups. The mean DFI Density Gradient Method mean was 4.40 ± 3.71, the Swim-up Method mean was 4.53 ± 3.46 and Magnetic Activated mean was 12.04 ± 5.55. there was Statistically significant (P<0.05). Similarly, HDS Type Swim up Method means was 0.43 ± 0.12. Magnetic Activated mean was 0.15 ± 0.10 anthe d Density Gradient Method mean was 0.24 ± 0.07. there was statistically significant(P<0.05).

Table-3

Unprocessed		Mean	SD	P-Value
DFI	Sample	28.40	3.45	0.014 Sig
	Control	11.50	11.64	
HDS	Sample	2.59	.51	0.021 Sig
	Control	1.47	.71	

The above table gives a comparison of the unprocessed values and it was seen that DFI in both groups was a means of differences between the groups. The mean DFI in the sample was 28.40 ± 3.45 and in control 11.50 ± 11.64, there was statistically significant (P<0.05). Similarly, the HDS Type sample was 2.59 ± 0.51 and the control was 1.47 ± 0.71. there was statistically significant (p<0.05).

Discussion (discuss your results with past study)

Despite the general fall in human fertility, effective diagnostic techniques have not yet been developed by science. As of now, all IVF methods choose sperm based on their overall morphology rather than their nuclear content or DNA normalcy. In addition, the majority of urological operations primarily rely on conventional semen analysis to determine the efficacy of the treatment.

In this research, the DFI & HDS values are calculated for each method of DG, SU, and M for Oligoasthenospermic patients. The investigation showed that the outcomes of the conventional analysis and those of the molecular techniques differed significantly. The DG method showed the method that had the lowest DFI index. This showed that this method is more suitable for Oligoasthenospermic patients.

Since these patients have low sperm concentrations, it is important to choose a proper sperm washing method. According to previous work (20), sperm with intact DNA cannot usually be extracted from semen using density gradient centrifugation. The TUNEL assay, which is not a highly sensitive assay because it only detects fragments typically caused by endogenous nucleases, was employed in this work to identify DNA fragmentations (21). In contrast, flow cytometric examination of samples that had undergone density gradient processing to detect apoptosis showed how effective this technique was at removing apoptotic sperm (2). The use of flow cytometry shows the effectiveness of the DG method which correlates with the result obtained in this research.

Conclusion:

The comparison of different methods of DS, DG, and M using flow cytometry has been carried out for the semen sample collected from 40 participants. On looking at the results obtained, additionally, the value of the mean for the DG method is found to show low DFI which makes the mean value to be low and makes it is suitable for oligoasthenospermic patients.

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