Best Washing Method for Oligoasthenospermic Patients by Evaluating DNA Integrity by Using a Sperm Chromatin Dispersion Test

Balachandran Sivaraja Ganesan¹*, Sumathy Govindarajan²*

¹Department of Anatomy, CSI College of Dental Sciences and Research, Madurai, INDIA.
²Department of Anatomy, Sree Balaji Dental College and Hospital, Bharath Institute of Higher Education and Research (BIHER), Chennai, INDIA.

Corresponding author:
¹ganesan. sivaraja8@gmail.com,
²ravi_sumathy@yahoo.com

ABSTRACT

Background: Growing evidence points to sperm DNA fragmentation (DF) as a significant contributor to infertility. One of the most popular techniques to identify sperm DF is the Sperm Chromatin Dispersion test (SCDT), a new assay in semen. The SCDT is based on the idea that after acid denaturation and removal of nuclear proteins, sperm with fragmented DNA are unable to create the distinctive halo of dispersed DNA loops seen in sperm with non-fragmented DNA. As Oligoasthenospermic patients have low sperm concentration, these patients are infertile and opt for fertility treatments for getting a child. There are many steps in the processing of sperm during Assisted Reproductive Technology (ART), and one of the main steps is Sperm Washing. Aim & Objective: The main aim of this research is to determine the best sperm-washing method for Oligoasthenospermic patients by evaluating DNA integrity (DI) by using SCDT. Study Setting: Semen samples were obtained from 40 Oligoasthenospermic patients with progressive motility <32%, who underwent ART procedure in Sumathi fertility center Madurai between Jan-feb2022. Informed Consent was obtained from all men prior to the study. This research was approved by an institutional review board of Bharath University Chennai. Material and Methods: The semen sample was subjected to Swim up (SU), Density Gradient (DG), and magnetic method. After washing, the samples were cryopreserved in liquid nitrogen for a period of 3 months. After 3 months DNA damage is measured by SCDT. The CANfrag DF Test kit was used to measure the DFI. Statistical Analysis: IBM SPSS was used to evaluate the significance of the results. Results: When compared to SU, the DG method shows the high level of un-fragmented DNA (big halo) were statistically significant p<0.01. When compared to the magnetic-activated method, the DG method shows a high level of the big halo was statistically significant p<0.01 similary. No Halo(fragmented DNA) was highly found in the magnetic activated cell sorting method when compared with SU and DG methods were statistically highly significant p< 0.001 similarly fragmented DNA very less in SU and DG methods were statistically highly significant p<0.001. Conclusion: The primary findings of the research indicate that the DG method best method for ICSI and IVF to increase the success rate.

Keywords

Oligoasthenospermic, DNA, Chromatin

Imprint


1. INTRODUCTION

The primary function of sperm is to mix the genetic material (DNA) from one biological parent with the genetic material present in the other biological parent’s egg to produce offspring with a distinct genetic code. Chromosomes, or DNA molecules, are tightly wound into a nucleus, which takes up most of the space in the head of the cell. The neck and tail’s purpose is to help the sperm convey its priceless “cargo” as effectively as possible.

The genetic integrity of sperm is an important factor in fertility since DNA contains all of the instructions for an embryo’s development. Any sperm DNA damage may prevent the sperm from properly fertilizing an egg or from growing into a strong, healthy embryo. The problem with sperm DNA fragmentation (DF) is just that. In comparison to other semen metrics and sperm quality measures including sperm count, motility, and morphology, sperm DNA fragmentation is less frequently evaluated and, generally speaking, less understood.
A. DF and DNA Fragmentation Index (DFI):

When one or both of the sperm’s DNA strands are broken or altered in some way, it is referred to as sperm DF. This harm can happen at several stages of the sperm’s life cycle, including when it is being created in the testicles through a process of cell division known as spermatogenesis when it is being “stored” in the epididymis before ejaculation, or even after ejaculation.

Sperm DNA fragmentation test (DFT) kits are essential since sperm DF is not assessed as part of a conventional semen study. The percentage of sperm in a specific semen sample with fragmented DNA is known as the DFI. An individual’s sperm contains genetic damage to a greater extent when their DF index is higher. Although specialists can’t agree on exact cut-off points, a DNA fragmentation score of over 30 to 50 percent is typically regarded as significant and may have an influence on fertility. Even individuals with a DNA fragmentation score of 15–30% may occasionally have subfertility, particularly if other aberrant semen parameters are present (such as low sperm count, poor sperm motility, or morphology), if the female spouse is older [1]. However, as more information about the connection between sperm DF and birth rates become available, there is a growing consensus among specialists that sperm genetic integrity may be one of the most crucial elements for male fertility. As a result, kits for testing the DF of sperm are especially helpful while attempting to get pregnant.

B. DF Testing:

Sperm DF testing is the most trustworthy way of determining the DFI. The sperm DFI, which measures the consistency of the sperm chromatin (SC), is one of many factors that influence whether or not a pregnancy will be successful (DFI). A high DFI prevents both conception and fertilization [2–6]. Therefore, while choosing the optimal assisted reproductive technology (ART) procedure for couples who have repeatedly failed to conceive, practitioners are aware of the significance of DFI in diagnosing male infertility. Several methods are currently available to evaluate DFI. Two of these diagnostic methods that depend on the SC’s denaturing power are the sperm chromatin structure assay (SCSA) and sperm chromatin dispersion Test (SCDT) kits.

SCSA uses a predetermined flow cytometry approach and program called SCSAsoft to provide a highly reproducible DFI measurement, which makes it the industry’s gold standard for quantifying sperm DF [7]. SCDT kits are light microscopy tests requiring a technician for a DFI based on either the presence or absence of a dispersal halo encircling the fragmented or non-fragmented sperm, respectively. Around fifty to five hundred sperm are counted in each sample. SCSA can be replaced by a number of SCDT kits at a reduced price.

The owners of SCDT have established a prediction criterion for infertile men between 20 and 27 percent for statistical categories of sperm fertility in published studies [8, 9]. It has been found that the success of fertilization is affected by a threshold between 17 and 18 percent [9, 10] The SCDT Halo sperm kit is the only one that uses this limiting value; it is not a rule that all SCDT must follow. SCSA criteria are currently used, however, there isn’t much consensus on when a given ART technique should be selected based on FI results on the DNA obtained with a specific SCDT kit. Researchers compared the DFI generated by SCSA and SCDT have revealed both congruence and incongruence between the 2 tests, for instance, a greater DFI was acquired when using an SCDT kit, which adds an additional degree of complexity [8, 11, 12].

C. Oligoasthenospermia and ART:

It concerns sperm count and sperm motility, two semen parameters. When the sperm concentration is less than 15 million/ml, the term Oligozoospermia is used. It is a disease of sperm that develops when sperm concentration is low. Oligospermia can be referred to as either. Or Oligozoospermia. In a laboratory, the number of spermatozoa in the ejaculate as a whole and the sperm concentration per millilitre are assessed. A concentration of 15 million sperm/ml and a minimum value of 39 million ejaculated sperm/ml have been established by the WHO. Oligospermia in men has no symptoms. This indicates that men can only be diagnosed with this change when they attempt to have a family but are unsuccessful [13].

It is exceedingly challenging to get pregnant naturally when there is oligoasthenozoospermia due to the altered semen characteristics. But it’s not impossible, either. Pregnancy is possible as long as there is one motile sperm in the ejaculate. The best course of action is case-specific. The great majority of couples may choose to use ARTs and visit a reproductive clinic in order to start a family. The most common method
of treatment to induce pregnancy is in vitro fertilization. The best sperms are chosen and combined with the eggs, which have already been removed via follicular puncture, in a laboratory setting. After that, the sperm fertilizes the egg to produce embryos, which are then placed in the woman’s womb after being cultured. An ICSI operation will be necessary for more serious situations. The procedures are the same as for IVF, except in this instance the specialist uses microinjection to choose and place the sperm within the egg. Numerous high-quality studies have shown that intrauterine insemination (IUI) is the first line of treatment for unexplained moderate male factor infertility and poor chances of spontaneous conception [14, 15]. For patients who opt for ARTs, the sperm needs preparation before it can be used. For this purpose, there are different sperm-washing techniques involved which is going to be the main focus. The different sperm-washing methods are discussed in the next section.

D. Sperm Washing Methods:

The different sperm-washing techniques are discussed here. First, the spermatozoa may be chosen using a process called “swim up (SU)” based on their capacity to swim. The culture medium is applied in layers over the liquefied semen in this method. Motile spermatozoa are introduced into the culture. The top layer of the stacked medium is then carefully removed for further use [16].

The utilization of density gradient (DG) is the second way of spermatozoa selection. The density column is placed on top of the pipetted semen sample, which is subsequently centrifuged. Spermatozoa are separated using DG centrifugation based on density. In this manner, the motile spermatozoa with normal morphology can be chosen and aspirated for further usage from the solution with the greatest gradient concentration [17]. In assisted reproductive procedures, DG centrifugation has been a common method for sperm preparation. Freshly taken semen specimens have been centrifuged on a Percoll gradient ranging from Forty and Ninety with excellent extraction [18]. Percoll was taken out of clinical human usage in late 1996. Covalently bonded hydrophilic silane-stabilized silica that was offered under a number of several brand names took the position of this product.

The third is the Miltenyi Biotec invention of Magnetic-activated Cell Sorting (MACS), a technique for dividing different cell populations according to their surface antigens (CD molecules). In positive selection, after removing the magnetic column from the magnetic field, the cells that expressed the desired antigen(s) and had adhered to the magnetic column are washed out and transferred to a different vessel. This technique is helpful for isolating a certain cell type, like CD4 lymphocytes. When using negative selection, the antibody is directed against a surface antigen that is known to be present in unimportant cells. The fraction that passes through the column after the cells and magnetic nanoparticle solution is applied to it is collected because it includes almost no cells that express the undesirable antigens [19].

Now that the basic parameters that are to be involved in this research are looked into, the study here focuses on Oligoasthenospermic patients. Since these patients are going for ART, the study here is to determine the best sperm-washing that is able to preserve the DNA Integrity by determining the DFI using the CANfrag kit.

II. MATERIAL AND METHODS:

A. Material:

Sperm wash media (VITROMEDia SAR HEALTH)
Sperm freeze media (VITROMEDia SAR HEALTH)
Magnetic Activated Cell Sorting (Canfrag), liquid nitrogen, and light microscope. Semen samples were obtained from 40 Oligoasthenospermic patients with progressive motility <32%, who underwent ART procedure in Sumathi fertility center Madurai between Jan-feb2022. Informed Consent was obtained from all men prior to the study. This research was approved by the institutional review board of Bharath University Chennai. SBDCH/IEC/02/2017/09. Briefly, the semen sample was subjected to SU, DG, and magnetic methods. After washing, the samples were cryopreserved in liquid nitrogen for a period of 3 months. After 3 months DNA damage is measured by SCDT.

CANfrag Principle and working:

To find DF in human sperm, use the CANfrag DF Test kit. This kit is based on an SCDT test. The kit’s operation relies on the denaturation of DNA to evaluate and distinguish between intact and fragmented DNA in spermatozoa. Untreated spermatozoa are sandwiched between two layers of base agarose and an inert top microgel. These layers are then subjected to acid treatment, which causes DNA breaks to form
constrained single-stranded DNA patterns. After that, a progressive lysis procedure is offered to remove the nuclear proteins. The halo and core are visible under a bright field microscope after dye staining. Large halos of intact DNA loops surround the center core of the sperm that has little or no DNA breakage. Sperm containing DNA fragments either do not create a halo at all or form a very small halo. Male infertility may be brought on by DNA fragmentation in sperm, which cannot be identified by standard semen measures such as sperm concentration, motility measurement, and morphological evaluation. This test is easy to interpret, fast, reliable, and reproducible.

B. Three methods for sperm washing:

SU METHOD:
After the semen has liquefied (usually 30 minutes at 37°C), 1 mL aliquots of semen are placed in 5 mL tubes that have labels (Falcon TUBE) and softly layered with 2mL of media (Sperm wash media) ALLOW TO CEN- TRIFUGE 1800rpm for 10 minutes. After centrifugation discards the supernant solution without disturb- ing the pellet. Add 5ml of flushing media and mix with pellet gently; centrifuge 1500rpm for 10 minutes and discard the supernant solution without disturbing the pellet. Add 2ml of IVF media and mix with pellet gen- tly, centrifuge 1200rpm for 10 minutes and discard the supernant solution. Incu- bate tubes at 37°C for forty-five to sixty min to allow increasingly motile sperm to swim into the overlying medium. Carefully add 0.5ml IVF media. Finally, ex- amine motility and count under a microscope.

DG METHOD:
Prepare 80/40 gradients in a 14ml tube, careful- ly overlay liquefied semen sample, and centrifuge 2000rpm for 20 minutes. Retrieve the gradients with caution, being careful not to dislodge the pellet. Mix the pellet with a 5ml flushing media centrifuge at 1500rpm for 10 minutes. Carefully remove the super- nant solution without disturbing the pellet. Mix the pellet with IVF media 2ml centrifuge 1200rpm or 8 minutes, discard the supernant solution. Carefully add 0.5ml IVF media, For forty-five to sixty min, in- cubate tubes at 37 around thirty seven°C to encourage increasingly motile sperm to swim into the medium overlaid. Finally, check count and motility under a mi- croscope.

MAGNETIC-ACTIVATED CELL SORTING METHOD
Magnetic-activated cell sorting (MACS) distinguishes between apoptotic and non-apoptotic sperma- tozoa. During apoptosis, phosphoryl serine residues are translocated from the spermatocoea’s intermem- brane space to its outer membrane (programmed cell death). Despite having a strong affinity for Phospha- tidylserine, Annexin V is not able to cross the intact sperm membrane. MACS is used to separate dead and apoptotic spermatozoa using colloidal super-para- magnetic beads (fifty nm in dia) linked to very par- ticular antibodies to Annexin V. Annexin V binding to spermatozoa suggests that the sperm membrane integrity has been disrupted.

C. Cryopreservation protocol (CP):
To prevent osmotic shock, 1 ml of sperm freezing media (glycerol cryoprotectant) was gradually added to the post-wash semen sample before it was transferred to the cryovials. The sample underwent two rounds of cooling: ten min at ambient temperature and ten mins in a Four degree C refrigerator. The sam- ples were then frozen using static vapour cooling be- fore being submerged in liquid nitrogen (-196 C).

D. Thawing protocol:
The cryovials were taken out of the liquid nitrogen and put at ambient temperature after CP. Small water droplets begin to collect on the cryovials’ outer surface as a result of the sample’s abrupt change in tempera- ture. Sweating is the term for this phenomenon. The entire sample was then taken and transferred to an equivalent volume of sperm wash media, which was then centrifuged for five minutes at 1500 rpm. The pellet was coated with IVF medium after the super- nant was removed. With the aid of a Makler cham- ber, the supernant solutions were aspirated to examine their motility.

Procedure:
The following are the steps followed:
1. place the provided Agarose Gel tube in float and incubated it in boiling water at 90c-100C for 2 minutes or until the gel melts. Then cool down this tube at 37 degrees c for 5 minutes,
2. Add 40μl of a semen sample from the sample preparation tube to the melted Agarose Gel tube and mix gently to avoid bubble formation.
3. Place 150μl of suspension on the coated slide, cover it with provided cover slip by avoiding air bubbles and transfer the slide at 4 degrees to 8 degrees c for 5 minutes to solidify the gel.

4. Take the slide out, and then gently remove the coverslip by sliding it off.

5. Place the slide on an even surface and overlay 1ml of Acid Denaturation. Incubate at 22 degrees c for 7 minutes and drain the solution completely after incubation.

6. Overlay 1 ml of lysis solution and incubate for ten min at room temperature. Drain the solution completely after incubation.

7. Gently wash the slide with 20 ml of distilled water with the help of a syringe or dropper by placing the slide in a vertically slanted position.

8. Place the slide on an even surface and sequentially dehydrate the slide by overlaying 1ml of dehydrating solution 1, dehydrating solution 2, and dehydrating solution 3 and incubate for 2 minutes each. then completely drain the excess solution.

9. Prepare working stain solution by mixing 300μl of stain and 300μl of dilution buffer.

10. Stain slide with 500μl of working stain solution.
RESULTS:

The results of SCDTT of sperm cells in three different technique separation techniques are presented in Table 1 below and in the graph.

The quantitative analysis of the SCDTT is shown in Table (1). The percentage of halo present surrounding the sperm head is used to express the values, which are presented as mean SEM. One-way ANOVA was used to examine the data, and Tukey’s test was used for multiple comparisons across groups. SU stands for SU, DG for DG, and MACS for Magnetic Activated Cell Sorting. Comparisons with SU, DG, and MACS are indicated by the symbols @, $, and, respectively. Statistical significance was attained at P values of * 0.05, ** 0.005, and *** 0.001.

The quantitative evaluation of the SCDTT is shown in the graph above. The various halo forms demonstrate the degree of DNA integrity. DNA that isn’t fragmented has a large, medium, or small halo, while DNA that doesn’t have a halo is fragmented. The percentage of halo present surrounding the sperm head is used to express the values, which are presented as mean SEM. One-way ANOVA was used to examine the data, and Tukey’s test was used for multiple comparisons across groups. SU stands for SU, DG for DG, and MACS for Magnetic Activated Cell Sorting. Comparisons with SU, DG, and MACS are indicated by the symbols @, $, and, respectively. Statistical significance was attained at P values of * 0.05, ** 0.005, and *** 0.001.

When compared to the SU, the DG method shows a high level of unfragmented DNA (big halo) was statistically significant p<0.01. When compared magnetic-activated method, DG the method shows the high level of the big halo was statistically significant p< 0.01. Similarly, No, Halo(fragmented DNA) highly found in the magnetic activated cell sorting method when compared with SU and DG methods were statistically highly significant p< 0.001 similarly fragmented DNA very less in SU and DG methods were statistically highly significant p<0.001. Small halo for all 3 methods values are identical so statistically not significant.

CONCLUSION:

The main purpose of this research was to present a comparison to determine the best washing technique for the sperm for patients who are undergoing ART. For this purpose, the CANfrag kit was used to determine the DFI. The sperm-washing washing methods
were SU, DG and MACS method. The primary findings of the research indicate that the DG method best method for ICSI and IVF to increase the success rate. This the effectiveness of this method was determined from the presence of high levels of unfragmented DNA – Intact DNA indicated by the test in the form of big Halo. This is shown in the result. One thing to be noted is that, though the current findings indicate that DG is the best washing method, this is based on the CANfrag kit and for this group of patients, and there is scope for future researchers to choose the different category of patients or use different testing kits to see the concurrence of the results.

REFERENCES: