

EFFECTS OF CRYOPRESERVATION ON SPERM DNA FRAGMENTATION AS ASSESSED BY SPERM CHROMATIN DISPERSION TEST IN NORMOZOOSPERMIC EJACULATED SPERMATOZOA

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ABSTRACT

BACKGROUND

Standard semen analysis for male infertility evaluation is inadequate. Sperm DNA integrity is vital for fertilization. Sperm function testing incorporating the assessment of sperm DNA integrity is critical since many cases of male infertility otherwise get labelled as "unexplained". Sperm chromatin dispersion (SCD) is a simple and easy test to assess Sperm DNA Fragmentation (SDF). With advancements in the ART, there is wide spread use of cryopreservation for fertility preservation. Freeze-thaw is reported to induce DNA damage to sperm cells. Present study is on effects of cryopreservation by analysing pre-freeze and post thaw SDF of semen samples by SCD.

The aim of the study is to evaluate the effects of cryopreservation on sperm DNA fragmentation (SDF).

The objective of the study is to assess the SDF as measured by the sperm chromatin dispersion (SCD) test on pre-freeze and post thaw cryopreserved semen samples.

MATERIALS AND METHODS

Single centre prospective cohort study.

100 normozoospermic male volunteers of infertile couple analysed for pre-freeze and post -thaw SDF by advanced version of SCD Halosperm[®] kit.

RESULTS

Significant increase (< 0.001) in the percentage of SDF in post thaw semen samples vis-a-vis pre-freeze samples was observed.

CONCLUSION

Cryopreservation / freeze - thaw of semen induces a slight but significant percentage of sperm DNA fragmentation (SDF). Hence it has a bearing on selection of ideal ART procedure.

KEYWORDS

Cryopreservation of Sperms, Sperm DNA Fragmentation (SDF), and Sperm Chromatin Dispersion (SCD) Test.

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BACKGROUND

Infertility affects 8 and 12% of couples. Male factors alone account for 20–30% of infertility and up to 50% of cases overall.^{1,2} Male factor contribution to the fertilisation is important.³ Standard semen analysis (WHO)⁴ alone cannot evaluate sperm DNA integrity.^{5,6} The integrity of sperm DNA is being recognized as a new parameter of semen quality and fertility potential. Morphologically normal sperms as assessed by Strict criteria may actually show DNA

fragmentation (DF). Couples on infertility treatment showed that 15.9% of normal sperm evaluated by high magnification microscopy had DNA fragmentation.⁷ Sperm function testing sperm DNA integrity and fragmentation correlates with fertility success both naturally and by artificial reproductive treatments (ART).^{3,8} Data from several studies suggest that infertile men have significantly greater amounts of DNA damage than fertile men. Similar finding is observed in the male partners of couples experiencing recurrent miscarriage. Unlike somatic cells, sperm chromatin is very compact and stable.⁹ Factors like cancer, infections, varicoceles,^{10,11,12} smoking and radiation^{13,14} cause sperm DNA fragmentation (SDF). Cryopreservation / freeze-thaw induced osmotic & thermal injury, reduction in sperm motility and DNA damage are well recognised observations.^{15,16,17} Cryopreservation of spermatozoa is widely used before vasectomy, therapy for malignant diseases, or surgical infertility treatments as well as for non-malignant conditions like diabetes, kidney

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disorders, and autoimmune diseases, the treatments of which may induce testicular damage.¹⁸

SDF analysis can be broadly categorized as direct and indirect assays. Direct ones are the 'Comet' and 'TUNEL' (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) assays and indirect ones are sperm chromatin structure assay (SCSA). The direct assays identify actual breakages in the DNA, while the indirect assays analyse the relative proportions of single and double stranded DNA within the sperm after acid denaturation.³ Extensive research on human semen samples, showed that SCSA is a very powerful technique (3) but requires expensive instrumentation, labor intensive, and necessitates the use of enzymes whose activity and accessibility to DNA breaks may be irregular. Hence, it may be best suited for research purposes rather than routine diagnostic use in the clinical andrology.¹⁹

DNA breakage detection-fluorescence in situ hybridization (DBD-FISH) is another procedure in which the restricted single-stranded DNA motifs generated from DNA breaks can be detected and quantified.²⁰ The Sperm Chromatin Dispersion (SCD) test, a relatively newer indirect method was developed to detect spermatozoa with increased amount of DNA fragmentation. The underlying principle of this test is that the intact unfixed spermatozoa are embedded in an inert agarose gel matrix on pretreated slide and exposed initially to acid denaturation of sperm cells with fragmented DNA followed by exposure to lysing solution that removes nuclear proteins. Normal sperms in the absence of massive DNA breakage produce nucleoids with large halos of spreading DNA loops around the nuclei by specific DNA fluorochromes and a fluorescence microscope. However, the nucleoids from spermatozoa with fragmented DNA either do not show a dispersion halo or the halo is minimal. Thus, enabling to detect spermatozoa with increased amount of DF.^{3,21} An advanced SCD test was developed two year after as a kit, Halo sperm.^{3,19}

In the SCD protocol, the sperm nucleoids may be visualized using fluorescence microscopy, after staining with a DNA specific fluorochrome (e.g., 6-diamino-2-phenylindole -DAPI) or with bright-field microscopy after Diff-Quik (Dade Behring, Switzerland) staining. The SCD protocol was good for assessment of halos using fluorescence microscopy but was inadequate for bright-field microscopy after Diff-Quik staining. The new SCD available as Halosperm[®] seems to have a slight but significantly higher sensitivity for detecting SDF (2.16% mean difference) than the SCSA. The results

suggest that the SCD test could be a good and cost-effective alternative to the SCSA.⁶ Measurement of sperm DNA fragmentation has a place in ART assessment to optimize the results under certain conditions, such as repeated ART failure, miscarriage, varicocele, inflammatory processes, or infection of the genital tract.²²

Effects of Cryopreservation

Cryopreservation is reported to inflict osmotic and thermal injury to sperms inducing changes in sperm morphology such as damage to mitochondria, acrosome, and sperm tail as well as dramatic drop in sperm motility.²³ Freeze- thaw process also decreases chromatin stability compromising DNA integrity. This was confirmed by Donnelly et al¹⁵ who demonstrated that an increase in single-strand DNA fragmentation during cryopreservation of human sperm inducing severe alterations in seminal parameters. Sperms may lose 25% to 75% of motility or become nonviable following cryopreservation, and this is attributed mostly to osmotic stress and formation of lethal intracellular ice crystals. Double-strand DNA fragmentation is more severe than single-strand DNA fragmentation and its effects on fertilization and embryo development are more severe because these types of breaks are more difficult to repair. These types of lesions are thus more pro mutagenic and may lead to important alterations to the embryo.⁷ With the advancement in ART, cryopreservation is being used increasingly irrespective of sperm concentration,²⁴ necessitating an understanding of the effects of cryopreservation on sperms.¹⁷ The present study therefore is to ascertain SDF by SCD on pre and post cryopreserved semen samples.

Aims and Objectives

- To study the effects of cryopreservation on Sperm DNA Fragmentation (SDF)
- To assess the SDF as measured by Halosperm[®] sperm chromatin dispersion (SCD) test on pre-freeze and post thaw cryopreserved semen samples.

MATERIALS AND METHODS

Single Centre prospective cohort of normozoospermic 100 male partners of infertile couple presenting at ART clinic were studied for one year after clearance from institutional ethical committee and obtaining written informed consent from voluntary participants satisfying the inclusion and exclusion criteria (see Box 1).

Inclusion Criteria	Exclusion Criteria
a) Normal physical examination b) Testes with normal volume c) Fresh semen samples collected by masturbation. d) Semen analysis of Male Partner: Normozoospermia (WHO 2010) e) Unexplained infertility and all other female factors of infertility	a) All male factor infertility: Azoospermia, Retrograde ejaculation, oligoasthanoteratozoospermia, immunological infertility. b) All other forms of collection of semen or extraction of sperms other than masturbation. c) Presence of Varicocele d) Isolation of any growth of organism on semen culture

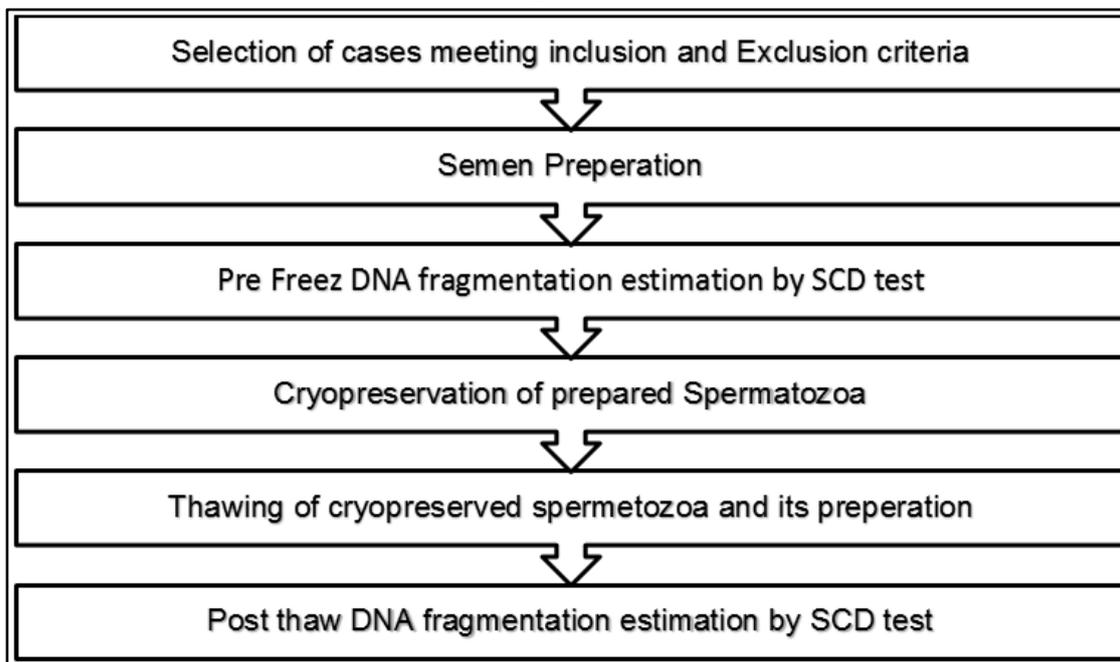
Box 1. Inclusion and Exclusion Criteria

Collection of Semen Samples and Sperm Preparation

Fresh semen samples were obtained by masturbation after an abstinence of 2 to 5 days in a private room near the ART Centre into a labelled wide mouthed sterile, nontoxic, plastic container. The collection, handling, and analysis were strictly as per protocols laid down in WHO manual 2010. Motion parameters were examined after mixing the sperm suspension and loading an aliquot into a Makler chamber. Sperm morphology was examined by oil-immersion microscopy by Strict criteria after staining with STAT III Andrology stain. Semen Preparations were carried out after liquefaction of the samples. We used bicarbonate buffered vitro life media for semen preparation. An aliquot of semen was retained and the remainder sample was prepared by the double density gradient and swim up (DDGSU) technique. Sperm morphology was reassessed. The prepared semen would then undergo Sperm Chromatin Dispersion test. After the DNA fragmentation test the semen sample would undergo cryopreservation. After 3-4 days spermatozoa were thawed after removal from the liquid nitrogen. The status of the thawed spermatozoa would be reassessed by the sperm chromatin dispersion test. Validity of the test is ensured by the procedure done by the same technician with the supervision of the two faculty members to avoid bias.

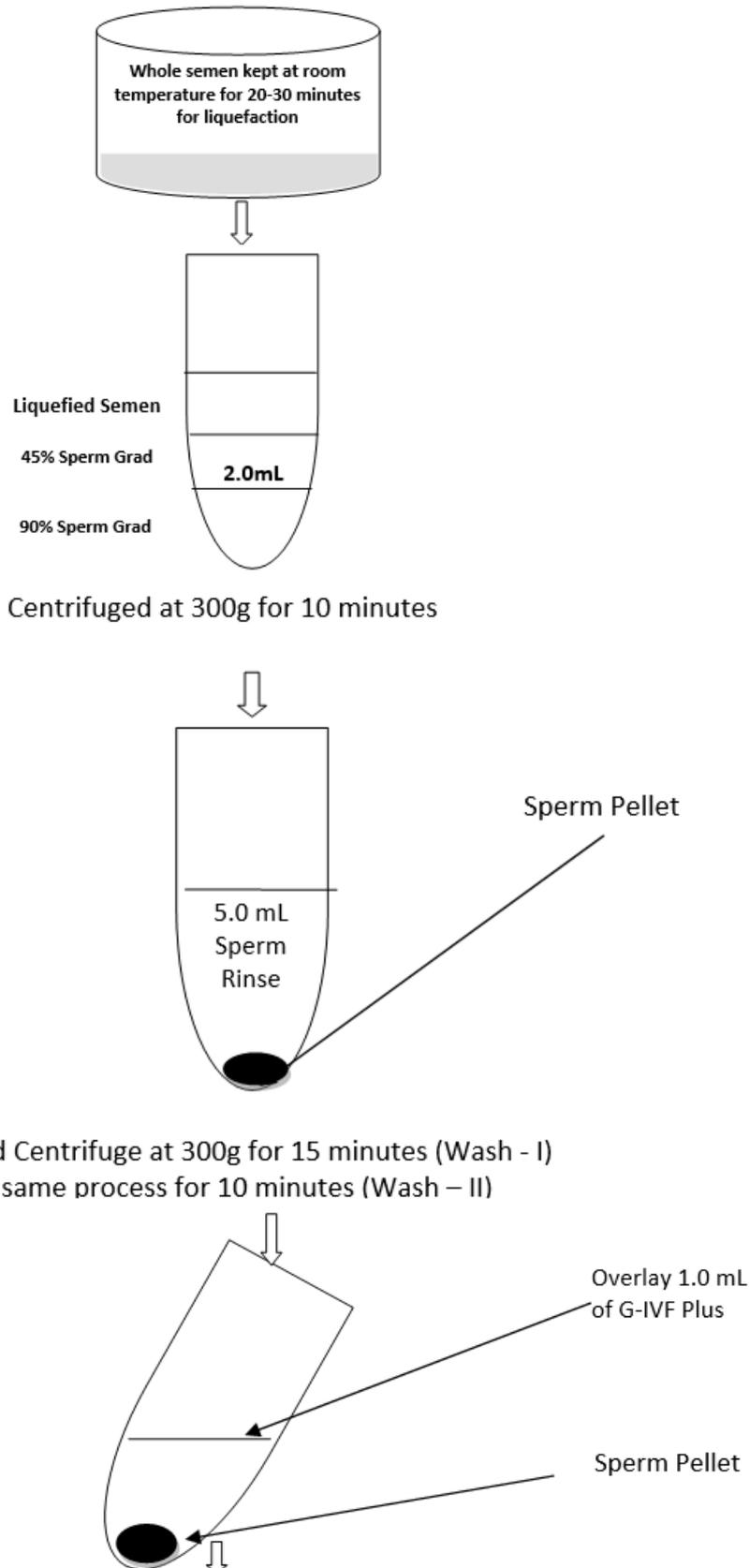
Double density gradient and swim up (DDGSU) semen preparation (Schematic diagram 2) was done as per standard protocol followed in this Centre. Sperm preparation medium was pre-equilibrated overnight. The lower layer (1.5 mL of sperm GRAD 90%) was first transferred into a conical centrifuge tube. The upper layer (1.5 mL of sperm GRAD 45%) was gently dispensed on top of the lower layer. A liquefied 2.0 mL semen sample was then placed on top of the upper layer. The tube was then centrifuged for 10 minutes at 300 g. In Wash I, the two top layers were carefully aspirated without disturbing the pellet and discarded. The pellet was transferred to new tube and re-suspended with 5 mL of Sperm Rinse medium and centrifuged again at 300g for 15 minutes. In Wash II, the supernatant was aspirated and discarded. The pellet was transferred to new tube and re-suspended with 5 mL Sperm Rinse medium and centrifuged again at 300g for 10 minutes. The supernatant was then removed, and the pellet was suspended in 1.0 mL of G-IVF PLUS medium, which was gently layered on top of the pellet, and the tube was inclined at an angle of 30° and incubated at 37°C for at least 15 minutes. After the incubation period, a sterile pipette was used to aspirate 0.5 ml of the top layer and transferred into sterile 5 ml round bottom tube and sperm concentration and motility were analysed in the recovered fractions.

Selection of cases meeting inclusion and Exclusion criteria



Schematic Diagram 1 Methodology

Schematic diagram 2 Double Density Gradient Swim Up (DDGSU) sperm preparation



Incubate at 6% CO₂ and 37°C for 15 minutes at 30° inclines with cap loose.
After incubation 0.5 mL removed for post wash analysis



Image 1. Halosperm® Kit

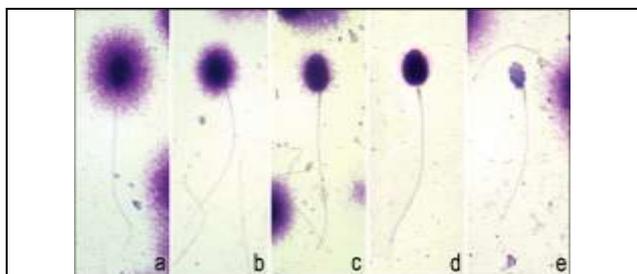


Image 2. Sperm Chromatin Dispersion Patterns Observed after Wright Staining. (a) Big Halo. (b) Medium Size Halo. (c) Small Halo. (d) Without Halo. (e) Without Halo and Degraded



Image 3. Cryo Preservation

Age (yrs.)	Age (yrs.)	Duration of Infertility (yrs.)	Duration of Infertility (yrs.)
Minimum	25	Minimum	04
Mean	32.8	Average	8.2
Maximum	40	Maximum	14

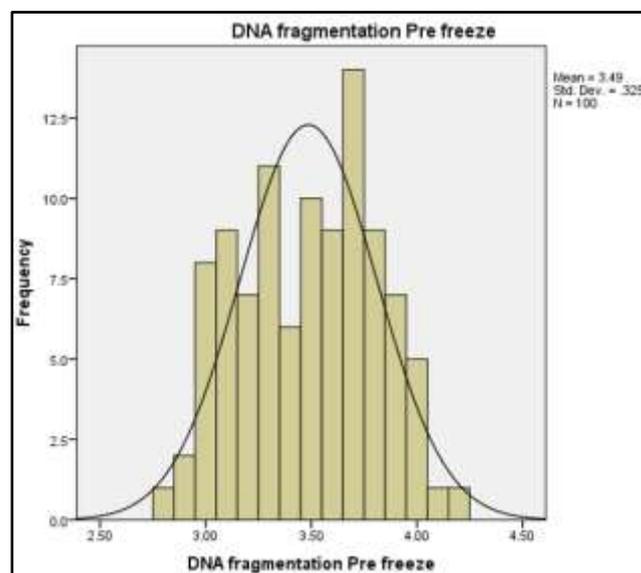
Table 1. Demography Profile of Participants

Serial No.	Aetiology	No. of Cases N=100	Percentage (%)
1.	Unexplained	34	34
2.	PCOD	24	24
3.	Endometriosis	15	15
4.	Bilateral Tubal Block	14	14
5.	Poor Ovarian Reserve	10	10
6.	Genital Tract Tuberculosis	03	03
7.	Total	100	100

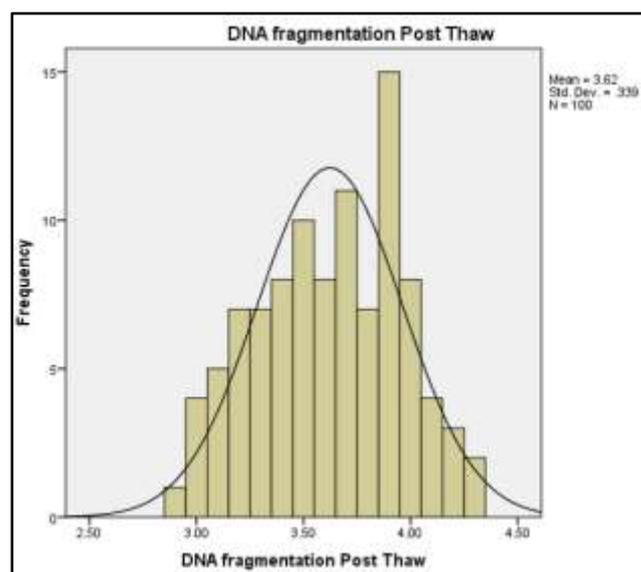
Table 2. Aetiology of Infertility

	DNA Fragmentation (%) Pre-Freeze (n=100)	DNA Fragmentation (%) Post -Thaw n=100
Mean	3.4860	3.6230
Median	3.5000	3.6500
Mode	3.70	3.90
Std. Deviation	.32474	.33900
Range	1.40	1.40
Minimum	2.80	2.90
Maximum	4.20	4.30

Table 3. Percentage (%) of Sperm DNA Fragmentation



Graph 1. Frequency Polygon: Prefreeze Sperm DNA Fragmentation



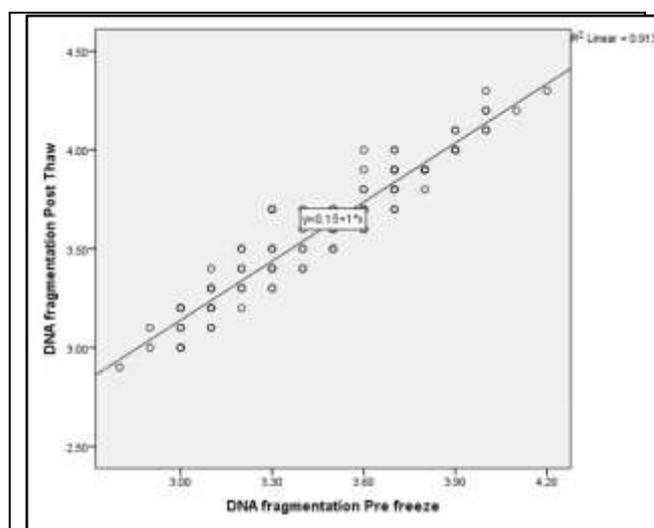
Graph 2. Frequency Polygon: Post-Thaw Sperm DNA Fragmentation

		Paired Differences					t	df	Sig 2-Tailed
		Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	Pre-Freeze SDF	3.4860	.32474	.03247	-0.157	-0.117	-13.679	99	.000
	Post-thaw SDF	3.6230	.33900	.03390					

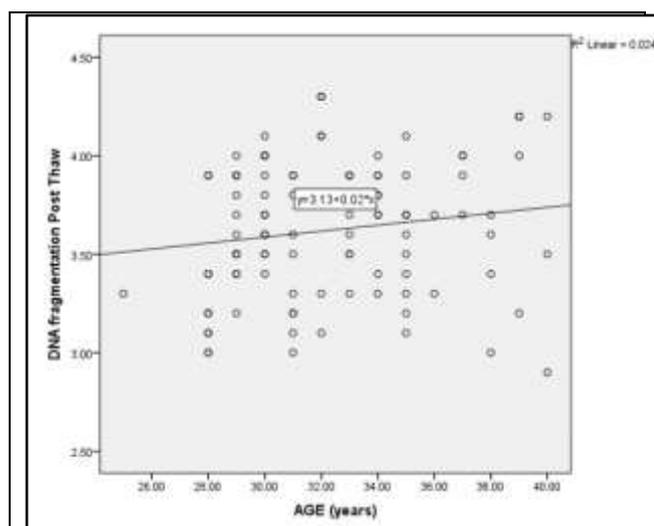
Table 4. Paired Samples T test

Pair 1		N	Correlation	Sig.
Pair 1	DNA fragmentation Prefreeze & DNA fragmentation Post Thaw	100	.955	.000

Table 5. Paired Samples Correlations (Pearson Correlation)



Graph 3. Scatter Plot (Bivariate): Prefreeze DNA Fragmentation with Post-Thaw DNA Fragmentation



Graph 4. Scatter Plot (Bivariate): Age in Years with DNA Fragmentation Post Thaw. Showing Effect of Age on DNA Fragmentation

SCD Test Protocol

SCD test was done using Halosperm ® kit (image 1). The underlying principle of the test is already outlined in the background section. An aliquot of a semen sample was diluted to 10⁶ /ml in phosphate-buffered saline (PBS). Eppendorf tube (one tube per one semen sample provided in the test kit with gelled aliquots of low melting point agarose) was placed in a water bath at 90°–100°C for 5 minutes to fuse the agarose, and then in a water bath at 37°C. 60 µ liter of the diluted semen sample was added to the Eppendorf tube after 5 minutes of incubation at 37°C, and mixed with the fused agarose. 20 µ liter of the semen-agarose mix was pipetted onto a pretreated slide with agarose provided in the kit, and covered with a coverslip. The slide was placed in the refrigerator (4°C) on a cold plate for 5 minutes to allow the agarose to produce a microgel with the sperm cells embedded within. The cover slips were gently removed by sliding off. Slide was immediately immersed horizontally in an acid solution, previously prepared by mixing 80 ml of HCl from an Eppendorf tube in the kit with 10 ml of distilled water and incubated for 7 minutes. The slide was horizontally placed and immersed in 10 ml of the lysing solution for 25 minutes. After washing 5 minutes in a tray with abundant distilled water, the slide was dehydrated in serially increasing concentrations of ethanol (70%, 90%, 100%) for 2 minutes each and then air-dried. The prepared slides can be stored at room temperature for several months in a tightly closed box in the dark. Slide was horizontally covered with a mix of Wright’s staining solution and PBS (1:1) for 5-10 minutes with continuous airflow. Slide was then washed in tap water and allowed to dry. Strong staining is preferred for easy visualization of the periphery of the dispersed DNA loop halos under bright field microscope. Percentage of SDF calculated by the formula below. Image 2 shows the halo and no halo images of sperms on a stained slide. Sperms with big halo (a) and medium halo (b) are taken as normal in the denominator. Sperms with Small halo (c), no halo (d) and no halo and degraded (e) are taken as fragmented and degraded in the numerator.

SDF=Fragmented + degraded/ total sperm counted x100

Sperm Cryopreservation (Image 3)

Prepared sperm concentrate was mixed with of sperm freezing medium (origio a/s) by adding slowly drop wise in to a container and shaking it for proper mixing and coating of cryoprotectant to the spermatozoa. Every prepared sperm concentrate would require about 1ml of media. Sperm concentrate and cryoprotectant mix is shifted in to cryovials. The mixture is left at room temperature for 10 min and then frozen by static phase vapor cooling for 30 min and then suspended in liquid nitrogen (-196⁰ C).

Thawing of Cryopreserved Sperm

Cryovial was removed from the liquid nitrogen after verification and identification and placed on a laminar airflow for 3 to 4 min. Then Cryovial was plunged in a water bath (37⁰ C for 10 to 15 min). Sample would display liquidity in the vial. Then it was kept at room temperature for 5-10 min. Sample was again mixed with equal volume of IVF media and the centrifuged at 300 g for 5 min and the formed pellet is taken out and resuspended in IVF media for further use.

The thawed sample was reassessed for SDF using SCD test (Halosperm® kit).

Outcome Measure

Pre-freeze and post thaw SDF were the primary outcomes studied.

Statistical Analysis

Statistical analysis was done by SPSS software version 20. Means were compared by paired sample t test. Correlation was evaluated by Pearson correlation coefficient. P value <0.05 was taken as significant.

RESULTS

100 male volunteers of infertile couple fulfilling the selection criteria were prospectively analysed for prefreeze and post thaw DNA fragmentation by SCD Halosperm® kit.

The mean, minimum and maximum age range of these normozoospermic (WHO Criteria) male volunteers was 32.8, 25 and 40 years respectively. The duration of infertility amongst the couples ranged from 04 to 14 years with an average of 8.2 years (Table 1).

Unexplained infertility (34%) was the commonest aetiology amongst the study population followed by PCOS (24%), Endometriosis (15%), tubal block (14%), poor ovarian reserve (10%) and Genital tuberculosis 3% in that order (Table 2).

Percentage of sperm cells with SDF is depicted in Table 3. The Minimum, maximum, median and standard deviation of percentage of SDF observed before freezing was 2.8%, 4.2%, 3.5% and 0.32474 respectively.

The Minimum, maximum, median and standard deviation of percentage of DF observed after thawing of the cryopreserved spermatozoa was 2.9%, 4.3%, 3.9% and 0.33900 respectively.

Frequency distribution of the Pre-freeze and post thaw percentage of SDF are depicted in respective frequency polygons. (Graph 1 & 2)

Paired samples statistics and paired samples t test are shown Table 4.

Two tailed paired sample t test done to compare pre-freeze and post thaw SDF show a significant difference ($p < 0.001$) in the scores of post thaw DNA fragmentation (mean=3.6230, standard deviation 0.3390) and pre-freeze DNA fragmentation (mean= 3.4860, standard deviation 0.32474); $t(99) = -13.679$ ($p < 0.001$) and 95% CI -0.157 to -0.117.

Pearson correlation as depicted in Table 5 shows an r value =0.955 which is significant ($p < 0.001$) clearly explaining the adverse effect of cryopreservation on SDF.

The scatter plot (Graph 3) of pre-freeze sperm versus post freeze DF clearly shows cryopreservation increases the DNA fragmentation.

Graph 4 shows the effects of age on DNA fragmentation. Age had no deleterious effect on the fragmentation of cryopreserved spermatozoa. The graph shows R2 linearity of 0.024.

DISCUSSION

Fertility preservation is of crucial importance when exposure to treatment with potentially sterilising agents or procedures is inescapable. Restoration of testicular function following chemotherapy or radiotherapy is unpredictable. As the chances of treatable childhood malignancies increase methods focusing upon preservation of fertility are necessary.²⁵ Cryopreservation is the only proven method to achieve this.²⁶ Recent advance in ART necessitated wide spread cryopreservation of semen not only for malignancies or such other conditions involving treatments that may cause progressive loss of testicular function (varicocele) but also for patients undergoing ART for severe oligospermia.^{27,28}

Cryo induced osmotic stress and lethal ice crystal formation and resultant severe alterations in seminal motion parameters are well recognized and documented. Loss of sperm motility may be to the tune of 25% to 75% of cells.^{6,29} There was no significant loss of motility in our study. Selection of normozoospermic participants, recovery of functionally superior quality of sperms by DDGSU method of semen preparation prior to cryopreservation and usage of cryoprotectant media could be responsible for this outcome. Thais Serzedello de Paula et al¹⁷ in their study had shown a loss of sperm motility to the tune of 25 to 75%. They had not used cryoprotectants and included Oligozoospermic semen samples. M.O Connel et al by R 123 staining could demonstrate mitochondrial injury mediated reduction in sperm motility during the process of freeze thaw.²³ Loss of sperm motility is not considered as an outcome in our study. SDF is the only primary outcome in the present study.

Median and standard deviation of percentage of sperm DNA fragmentation observed on pre-freeze and post thaw sample was 3.5%, 0.32474 and 3.9%, 0.33900. respectively. This was in agreement with the study by Robert

E et al which has shown DNA fragmentation of $4.0 \pm 1.0\%$ (2). The paired samples t test in our study show significant ($p < 0.001$) difference in the pre-freeze SDF vis-à-vis post thaw samples.

Pearson Correlation for DNA fragmentation for Pre-freeze and post thaw sample is ($r=0.955$) (p value < 0.001) clearly shows the significant negative effect of cryopreservation on spermatozoa in the form of DNA fragmentation. Our study is well supported by the similar study by Eilish T. Donnelly et al which concluded that Cryopreservation significantly affects the DNA integrity of infertile men where as it has no adverse effect on DNA integrity of fertile men.²⁴

Our study has shown that age less than 40 years per se does not affect DNA fragmentation. Graph 4 shows R2 linearity of 0.024. Demographic profile of participants in this study cannot be compared with other studies. Sperm DNA fragmentation is affected by many variables like infection, Varicoceles, stress and bad lifestyle which has been already proved by many studies.²⁵

CONCLUSION

Cryopreservation increases the DNA fragmentation level of washed sperm significantly.

Recommendations

Careful selection of post-thaw sperms for fertilization is important. Level of DNA fragmentation helps in selection of an ideal ART procedure. Further improvisation in the cryoprotectant technology may in future reduce SDF. Novel techniques like Intracytoplasmic Morphologically Selected Sperm Injection (IMSI) and Magnetic Activated Cell Sorting Techniques have come into practice for selecting sperm cells of uncompromised DNA integrity.

Limitations of the Study

1) Single centre study 2) Limited sample size. 3) Infertile men with normozoospermia only were selected. 4) Post wash samples by DDGSU method already selects sperm cells of intact DNA integrity hence there could be a selection bias.

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