Original Article

Evaluation of Sperm DNA Fragmentation after Cryopreservation in Ejaculated Spermatozoa

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Abstract

Introduction: Infertility has been declared as a public health concern by the World Health Organization. Infertility affects approximately 10%–15% of couples worldwide. Male factors contribute significantly to infertility approximately 35% of couples. Assessment of the integrity of sperm DNA is important in male infertility. Semen cryopreservation techniques as a measure of fertility preservation have been shown to increase DNA fragmentation. The main objective is to study the effects of cryopreservation on sperm DNA fragmentation in ejaculated spermatozoa. **Material and Methods:** The study was conducted in a tertiary care referral hospital for infertility during the period of January 01, 2013–March 31, 2014. A total of one hundred patients who met the inclusion criteria were included in the study. Sperm DNA fragmentation was done prefreeze and postthaw by sperm chromatin dispersion test. **Results:** Mean sperm count prefreeze was 56.6 million/ml (standard deviation [SD] = 22.5 million) of semen. Lowest concentration of spermatozoa in the study population was 25 million/ml of semen and highest concentration of spermatozoa in the study population was 120 million/ml of semen. Postfreeze concentration had mean of 66.1 million (SD = 22.4 million). DNA fragmentation in prefreeze was 3.5% (0.3%) and in postfreeze 3.6% (0.3%). There was statistically significant difference between prefreeze and postfreeze values both in sperm count and DNA fragmentation. There was a statistically significant correlation between age and postthaw DNA fragmentation. **Discussion and Conclusion:** Although cryopreservation increases the DNA fragmentation level of washed sperm significantly, this does not prevent us from utilization of cryopreservation facility because benefits far outweigh the adverse effects of cryopreservation.

Keywords: *Cryopreservation, DNA fragmentation, infertility*

Introduction

Infertility has been declared as a public health concern by the World Health Organization (WHO). This condition affects approximately 10%–15% couples worldwide. Male factors contribute significantly to infertility approximately 35% of couples.[1] Most of the Assisted Reproductive techniques (ART) programs do not evaluate the male extensively and just perform a routine semen analysis and semen culture.[2] However, in majority of the patients, the etiology of male factor cannot be established based on findings of standard semen analysis alone.[3,4] Recently, various medical assays have been employed for the assessment of sperm DNA fragmentation. Sperm chromatin is packaged very tightly as compared to chromatin in somatic cells. Different mechanisms exist which result in damage to the compact and stable structure of the sperm DNA.[5]

Relevant research into sperm DNA organization also attempted to establish the causes of sperm DNA fragmentation and propose mechanisms of DNA damage in spermatozoa. Varicoceles, systemic infections, male accessory gland infections, and cancer have been identified as important causative factors.[6-8] Environmental and lifestyle factors such as obesity, smoking, drugs, pollution, and radiation, have also been implicated.[9,10] It has been proposed that oxidative stress, i.e., generation of the reactive oxidative species is an important mechanism responsible for the nicks and breaks in the sperm DNA. It may occur during spermatogenesis or during the prolonged storage of spermatozoa within the epididymis. Semen cryopreservation has been offered as a method of fertility preservation in men

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undergoing vasectomy prior to the operative procedure and in cancer patients. Semen cryopreservation can also be performed in obstructive azoospermia and congenital bilateral absence of vas deferens by surgically retrieving of sperms from epididymis followed by their use in *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI) cycles. Semen cryopreservation may also be done in diseased conditions such as diabetes, kidney disorders, and some autoimmune diseases as gonadotoxic damage have been reported in these conditions.[11] Cryopreservation is very essential in cases of intrauterine insemination (IUI) donor because serological markers for the diseases such as HIV and hepatitis B surface antigen have been detected after the fresh sample IUI donors which were negative at the time of insemination. Cryopreservation takes care of the window period and repeat tests for virological markers can be done at the time of insemination.[12]

Relevant research studies on male infertility have indicated that some of the DNA fragmentation changes may be iatrogenic. Semen cryopreservation techniques as a measure of fertility preservation have been shown to increase DNA fragmentation.[13,14] It is an established finding that considerable decrease in overall sperm motility is observed postcryopreservation which is predominantly attributed to osmotic stress and thermal damage to the cell membranes and organelles including intracellular ice crystal formation. Cryopreservation also significantly decreases chromatin stability with detrimental consequences to DNA organization and compact packing.[15]

Standard semen analysis done for the evaluation of male partner in infertility mainly focuses on sperm concentration, motility, and morphology. The result of semen analysis fails to comment on sperm DNA integrity. Hence, a need was felt to develop tests for detecting the sperm DNA fragmentation. There are two basic types of assays for sperm chromatin fragmentation: direct assays, for example, the "Comet" and "TUNEL" (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphates nick end labeling) assays and indirect assays, for example, the sperm chromatin dispersion (SCD) assay or sperm chromatin structure assay (SCSA). The direct assays such as comet assess actual nicks and breaks in the DNA, whereas the indirect assays such as SCSA detect the relative proportions of both the single- and double-strand DNA breaks within the sperm following the initial acid denaturation treatment. Most of the relevant studies carried out to investigate the etiology of male infertility demonstrated that infertile males have significantly more degree of sperm DNA fragmentation as compared to fertile males. It has also been investigated that these findings also occur commonly in male partners of infertile couples diagnosed to have repeated spontaneous abortions. However, no statistically significant correlation exists between levels of sperm DNA fragmentation and reproductive outcomes in natural or assisted reproductive technologies cycles.[5]

In the modern-assisted reproductive techniques (ARTs) practice, semen cryopreservation is being increasingly offered to infertile men irrespective of the results of sperm concentration found on standard semen analysis.[8] Moreover, semen cryopreservation is increasingly used in fertility preservation in cancer, thus necessitating research on the consequences of freezing on spermatozoa.[15] Therefore, the present study set out to study the consequences of cryopreservation on sperm DNA fragmentation as evaluated by SCD test on pre- and postcryopreserved semen samples.

Material and Methods

The study was conducted in a tertiary care referral hospital for infertility during the period January 01, 2013–March 31, 2014. All patients were screened for inclusion and exclusion criteria. The inclusion criteria were patients undergoing treatment for infertility with male partner having the following characteristics: normal general and systemic examination, normal volume of testes, absence of varicoceles, and sterile semen cultures. The exclusion criteria were patients of azoospermia, oligospermia, and oligoasthenoteratozoospermia.

Sample size

The sample comprised 100 male partners of couples presenting with infertility. Written informed consent was obtained from all the participants and their spouses. The hospital ethical committee gave the approval for the study. The data were entered into MS Excel sheet and analyzed using Stata Statistical Software:13. College Station, TX: StataCorp LP. $P < 0.05$ was considered statistically significant.

Procedure

SCD test was used because it is a simple and reproducible test for the assessment of sperm DNA fragmentation. The steps of the procedure were reproduced from the method advocated by Jose Luis Fernandez *et al*. for the assessment of sperm DNA damage which was commercially available as Halosperm Kit.

Semen collection and initial assessment

1. After 2–5 days of abstinence, semen sample was obtained by masturbation into a sterile 100 ml plastic container
2. After allowing for liquefaction for 30–45 min in an incubator, a standard semen analysis was performed using light microscopy as per the WHO guidelines
3. Sperm concentration and motility were determined using a Maklers chamber (Sefi Instruments, Israel)
4. Sperm morphology was examined using 100 × objective of oil-immersion microscopy as per the WHO guidelines after staining with Wright's stain.

Preparation of semen sample

1. A small portion of semen sample was retained and the remaining sample was processed by the combination of double-density gradient and swim-up technique
2. Sperm morphology was reassessed
3. SCD test was performed on the processed semen
4. After the SCD test, the semen sample underwent freezing
5. After 3–4 days, frozen semen sample was thawed after removal from the liquid nitrogen
6. The status of the thawed spermatozoa was reassessed by SCD test.

Validity of the test was ensured by the procedure done by the same technician with the supervision of the two consultants to avoid bias involved in the procedure.

Procedure of sperm chromatin dispersion test

1. A small part of the processed semen sample was diluted to approximately 5–10 million/mL in phosphate-buffered saline (PBS).
2. Small aliquots of agarose gel in 0.5 ml Eppendorf tubes were included in the Halosperm kit
3. Eppendorf tubes were kept in a water bath at 90°–100°C for 5 min to fuse the agarose microgel, and followed by keeping in a water bath at 37°C for 5 min.
4. Sixty microliters of the diluted semen sample was added to the Eppendorf tube and mixed with the fused agarose microgel
5. Small aliquot of 20 µl was pipetted from semen-agarose mixture onto slides precoated with agarose provided in the Halosperm kit and covered with a coverslip
6. The slides were transferred to a cold glass plate in the refrigerator at 4°C for 5 min to allow embedding of spermatozoa within agarose microgel
7. The coverslips were carefully removed and the slides dipped horizontally in HCl solution. This solution was prepared by adding 80 µl of HCl in an Eppendorf tube to 10 mL of distilled water and mixed thoroughly. The resultant solution was incubated for 7 min
8. The slides were dipped in 10 mL of the lysing solution horizontally for 25 min. The slides were washing for 5 min with plenty of distilled water. Subsequently, the slides were dehydrated in increasing concentrations of ethanol (70%, 90%, 100%) for a duration of 2 min each and then air-dried naturally
9. Slides were stored in slide trays kept at room temperature
10. For conventional bright field microscopy in the improved SCD test (Halosperm® kit), slides were horizontally placed and covered with a mixture of Wright's staining solution and PBS (1:1) for 5–10 min
11. Slides were washed in running tap water and allowed to air dry. Good staining is a prerequisite to observe the dispersed DNA loops forming halos under 100X objective of the light microscope.

Prepared sperm concentrate was mixed with sperm freeze cryoprotectant by adding cryoprotectant into sperm concentrate container slowly dropwise and shaking the container for proper mixing and coating of cryoprotectant to the spermatozoa. Approximately 1 ml of cryoprotectant media was required for every processed sperm concentrate. Sperm concentrate and cryoprotectant mixture were shifted into cryovials. The mixture was kept at room temperature for 10 min and then cryopreserved by nitrogen vapor cooling for 30 min and then suspended in liquid nitrogen at −196°C.

Thawing of cryopreserved sperm

After checking the proper identity and details, cryovials were removed from the liquid nitrogen and placed on a laminar airflow for 3–4 min. Plunge the cryovial in a water bath (37°C for 10–15 min). Sample would display liquidity in the vial; it was then kept at room temperature for 5–10 min. Sample was again mixed with equal volume of IVF media and centrifuged at 2500 rpm for 5 min and the formed pellet was taken out and resuspended in IVF media for further use.

The thawed sample was reassessed for sperm DNA fragmentation using SCD test (Halosperm® kit).

Results

Demographic profile

Mean age of male partners who were subjected to DNA fragmentation was 32.8 years (standard deviation [SD] = 2.5 years) and ranging from 25 to 40 years. The duration of infertility varies from 4 to 14 years.

Average duration of infertility male partners who were subjected to DNA fragmentation was 8.2 years (SD = 1.4). The causes of infertility are as given in Table 1.

Most common etiology among the study population was unexplained infertility is about 34% and the least common etiology was genital tuberculosis about 3%.

Spermatozoa characteristics

Mean sperm count prefreeze was 56.6 million/ml (SD = 22.5 million) of semen. Lowest concentration of spermatozoa in the study population was 25 million/ml of semen and highest concentration of spermatozoa in the study population was 120 million/ml of semen.

Table 1: Causes of infertility

Etiology	Number of cases (%)	95% CI
Unexplained	34 (34)	25–44
Bilateral tubal block	14 (14)	7–22
PCOD	24 (24)	16–34
Genital tuberculosis	3 (3)	0.6–9
Endometriosis	15 (15)	8.6–23.5
Poor ovarian reserve	10 (10)	5–17.6

CI: Confidence interval, PCOD: Polycystic ovarian disorder

Postfreeze concentration had mean of 66.1 million (SD = 22.4 million). DNA fragmentation in prefreeze was 3.5% (0.3%) and in postfreeze 3.6% (0.3%). The spermatozoa were assessed at × 1000 magnification under oil immersion lens after staining with Wright stain [Figures 1 and 2].

There was statistically significant difference between prefreeze and postfreeze values both in sperm count and DNA fragmentation [Table 2].

There was statistically significant correlation between pre DNA fragmentation and post DNA fragmentation ($P < 0.001$) and the linear relationship between the two is depicted in Figure 1.

There was a statistically significant correlation between advancing age and postthaw DNA fragmentation. With 10 years increase in age, DNA fragmentation increased by 2%. The same is shown in Figure 2.

Discussion

Approximately 40%–50% of infertility problems are attributed to male infertility.[16] Infertile men having normal semen parameters of sperm motility and morphology may show DNA fragmentation. In addition, a relevant report of infertile couples undergoing assisted reproduction showed that 15.9% of normal spermatozoa separated by highly selective sperm selection technique of higher magnification microscopy had sperm DNA damage.[17] Compromised Sperm DNA integrity has been associated with diminished natural reproduction and pregnancy outcomes in IUI and IVF. High sperm DNA fragmentation negatively affects fertilization rate, embryo cleavage, clinical pregnancy rate and ongoing pregnancy rates in IVF.[18]

Evaluation of sperm DNA fragmentation may predict sperm quality and fertility potential. Many tests have been advocated to assess sperm DNA fragmentation, such as SCD test, the terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL), *in situ* nick translation, sperm adduct analysis, 8-hydroxy 2 deoxyguanosine levels, comet assay, and SCSA. Extensive research has shown that SCSA is a robust technique. However it requires expensive equipment, is labor intensive and not routinely done in andrology laboratory. Most of these tests are done in research settings.[19]

SCD test is a comparatively simple and reproducible test. It involves acid denaturation followed by embedding of the spermatozoa in an agarose microgel precoated on the slides. After initial acid denaturation, the nuclear proteins are removed by lysis solution resulting in unwinding of the DNA with a peripheral halo of dispersed DNA loops around the central core. Thus, sperm DNA fragmentation is indirectly reflected by size of dispersion halo and can be assessed using the bright-field microscope or a fluorescent microscope. SCD is a simple, accurate, and highly reproducible test.[19]

The improved SCD protocol commercially available as the Halosperm kit is claimed to have slightly higher sensitivity for detecting sperm DNA damage (2.16% mean difference) than the SCSA.[4] Measurement of sperm DNA fragmentation, though, not routinely recommended has a place in ART assessment to address idiopathic infertility and in cases of repeated ART failure, early pregnancy loss, varicoceles, and genital tract infections.[20]

Cryopreservation reduces motility due to osmotic and thermal injury to sperms including intracellular ice crystal formation. The stability of the sperm chromatin reduces during the cryopreservation resulting in sperm DNA fragmentation. This finding was reported by Donnelly *et al*. who showed a significant increase in single-strand DNA breaks during freezing of human sperm and cryopreservation induced severe alterations in semen parameters. He also concluded that double-strand DNA breaks are more severe, and its effects on pregnancy outcomes are detrimental because aging oocytes have

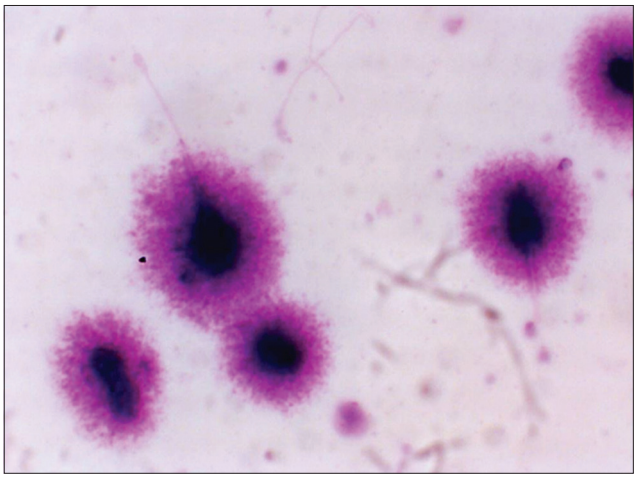


Figure 1: Spermatozoa seen at × 1000 under oil immersion

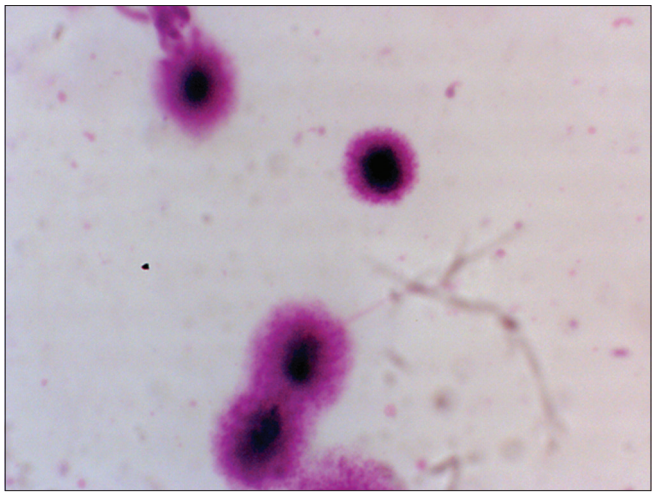


Figure 2: Spermatozoa seen at × 1000 under oil immersion

decreased capacity of repairing double-strand breaks. These breaks may be responsible for mutations in the embryo.[15] Cryopreservation has been reported to induce detrimental changes to sperm morphology including damage to cell membranes and cell organelles such as mitochondria and acrosome.[21]

Fertility preservation in males of reproductive age who have not planned their parenthood and recently diagnosed with cancer involves cryopreservation of several semen samples. Testicular damage in these patients is due to potentially gonadotoxic chemotherapeutic agents or radiotherapy. Moreover, restoration of normal spermatogenesis after chemotherapy and radiotherapy is poor. Modern assisted reproductive technologies focus on cryopreservation methods for maintaining a patient's fertility potential and achieving parenthood.[22]

Semen cryopreservation is the beneficial technique that gives these patients a chance to achieve pregnancy in the future and has proved to be a boon for young cancer survivors.[23] Cryopreservation of semen has thus been offered in diverse situations for patients prior to vasectomy, chemotherapy, radiotherapy, and also for patients with nonmalignant conditions. Recent advances in the field of assisted reproductive technologies (ART) like ICSI have broadened the scope of ART and have proved a boon for treating male infertility. Even oligospermic patients can achieve fertilization and therefore necessitating cryopreservation of semen with low counts.[24]

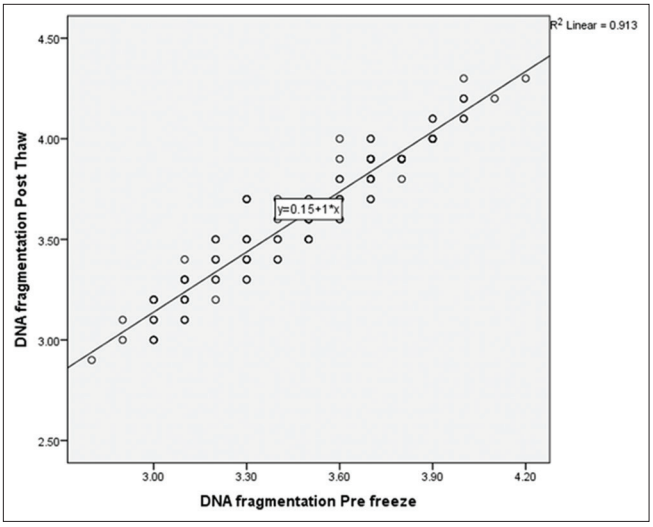
Cryopreservation results in detrimental changes in semen parameters and sperms may lose motility after cryopreservation mostly due to osmotic and thermal stress. The formation of intracellular ice crystals reported during the cryopreservation process has been implicated in damage to the plasma membrane and the membrane bound cell organelles including mitochondria and Golgi apparatus.[4,25] In our study, loss of sperm motility was 3%–5% which is very less compared to above studies, and this significant difference may be because we had selected normozoospermic patients and we had used cryoprotectant media as it was not used in the study by de Paula, *et al*. which had shown loss of motility from 25% to 75% and it had included cases with oligozoospermia and normozoospermia. Reduction in motility is due to the mitochondrial injury during the process of freeze-thaw as demonstrated by O'Connell *et al*. through R 123 staining of spermatozoa before and after staining.[21]

In our study, mean age of male partners who underwent evaluation of sperm DNA fragmentation was 32.8 years and average duration of infertility male partners who were subjected to DNA fragmentation was 8.2 years. These demographic characters could not be compared with other studies as our selection criteria's were different from other studies. Our study has shown that age <40 years *per se* does not affect DNA fragmentation as shown in our first graph [Graph 1] depicting effect of age on DNA fragmentation which shows R^2 linearity of 0.024. It is affected by many variables such as infection, varicoceles, stress, and unhealthy lifestyle which has been already proved by many studies.[22]

Most common etiology among study population was unexplained infertility is about 34% and the least common etiology was genital tuberculosis about 3% again these factors cannot be compared with other studies as our selection criteria's were different from other studies and aim of the study was not to ascertain the etiology of infertility.

Overall mean concentration of spermatozoa was 56.63 million/ml of semen [Table 2]. Median DNA fragmentation noticed before freezing was 3.5% with the SD of 0.32474, and postthaw, it was 3.6% with the SD of 0.33900. The levels of sperm DNA fragmentation in our study were in agreement with the study by Jackson *et al*. which has shown DNA fragmentation of 4.0 ± 1.0%.[16]

Pearson correlation for DNA fragmentation for prefreeze and postthaw sample is 0.955 which clearly explains



Graph 1: Relationship between pre- and post-DNA fragmentation

Table 2: Sperm count and DNA fragmentation

Serial number	Characteristic	Prefreeze (*n*=100)	Postfreeze (*n*=100)	*P*
1	Sperm count mean (SD)	56.6 (22.5)	66.1 (22.4)	<0.001
2	DNA Fragmentation; mean (SD)	3.5 (0.3)	3.6 (0.3)	<0.001

SD: Standard deviation

the adverse effect of cryopreservation on spermatozoa in the form of DNA fragmentation and the correlation is statistically significant 0.01 levels (two-tailed). Although the difference of 0.1% is not large, the median DNA fragmentation was statistically significant. Our study is well supported by a similar study by Donnelly *et al*. which concluded that cryopreservation significantly affects the DNA integrity of infertile men whereas has no adverse effect on DNA integrity of fertile men.[12]

Conclusion

Cryopreservation of semen is indicated for fertility preservation in young cancer patients undergoing gonad toxic radiotherapy and chemotherapeutic agents. It is also beneficial prior to performing a vasectomy and in certain medical conditions which cause loss of testicular function. In some situations, cryopreservation of semen samples is done as a backup for the intended use of the sample on the day of the IVF or the ICSI procedures. Although cryopreservation increases the DNA fragmentation level of washed sperm significantly, this does not prevent us from utilization of cryopreservation facility because benefits far outweigh the adverse effects of cryopreservation.

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Conflicts of interest

There are no conflicts of interest.

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