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Effect of Sperm Cryopreservation on Biochemical Profile and DNA Fragmentation: A Hospital Based Observational Study

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Abstract

Background: Sperm cryopreservation potential can be explored in diverse conditions to hamper future fertility. Reactive oxygen species (ROS) were among the prime candidate responsible for vulnerability of the sperms due to lack of intracellular antioxidants defense.

Aim: The study aim to investigate the effect of cryopreservation on sperm biochemical profile and DNA fragmentation.

Methods: This controlled observational hospital-based study was conducted on men participants (n=100). The semen specimens with normal sperm count and motility post fertility work up was collected in a sterile vial containing cryoprotectant and thereafter stored in a freezer having liquid nitrogen -196°C for 7 days at controlled rate. Sperm morphology, DNA fragmentation and motility along with malondialdehyde and total antioxidant capacity (TAC) were measured before and after cryopreservation.

Results: Semen specimens post cryopreservation demonstrated significant decrease in sperm motility, number and malondialdehyde level ($p<0.001$). There was a significant increase in DNA fragmentation and total antioxidant capacity levels ($p<0.001$).

Conclusion(s): Sperm cryopreservation may lead todeleterious variations of structure and function(s) due to generation of ROS that contribute in DNA fragmentation.

Keywords: Human sperm, cryopreservation, DNA fragmentation, total antioxidant capacity, sperm motility, malondialdehyde, sperm morphology

Introduction

Cryopreservation being an essential and consistently practiced procedure in assisted reproduction technology (ART) that hampers the sperm's vital functions due to cellular damage. Previously published studies advocated that freezing and thawing procedure with sperm adversely affect sperm motility and numbers along with DNA damage [1-7]. Intracellular ice crystals formation, cold shock, ROS generation and osmotic stress contribute to this damage during the cryopreservation procedure [8-9]. It is well known that the membrane and cytoskeleton elements were sensitive to temperature variations and thereby initiate cell injury and thereby affecting organelle vital functions. Impairment in the structural morphology is contributed due to disorganization of membrane fluidity during the cryopreservation procedure as studied by authors of previously publish study [8]. There are resilient of published evidences which are suggestive of ROS generation from the mitochondria during the procedure of freezing and thawing [10-12]. These free radicals could be one of the prime culprits of cellular and DNAdamage that further contribute in lowering of total antioxidant capacity in cryopreserved specimens [5, 13].

Sperm cell membranes are vulnerable to malondialdehyde and peroxidative damage, which is a catalogue of lipid peroxidation. Lower expression of antioxidant defense system or huge turnover of potential cellular oxidants vice-versa causes cellular oxidative stress. Apoptosis is another potential contrivance responsible for sperm DNA damage during the procedure of cryopreservation [14]. During the process of spermatogenesis, apoptosis initiate impairment in the cell membrane, decrease in cell volume and degradation of DNA. Another previously published study, authors found increased activity of caspases and DNA fragmentation in freeze thawed spermatozoa specimens [15]. Few studies also reported significant decrease in antioxidant activity in cryopreserved sperm [16-17].

The present work further aimed to investigate the effect of cryopreservation on total antioxidant capacity and free radical induced DNA fragmentation upon thawing of the cryopreserved specimens. Furthermore, the levels of malondialdehyde were also measured in the present study.

Material and methodsstudy design and participants

Healthy study participants (n=100) aged between 18 to 40 years were enrolled from the Embryology laboratory. Men with azoospermia, severe oligozoospermia or leucocytospermia were excluded from the study. Written informed consents from all eligible subjects were obtained before recruitment. The Ethics Committee, of Pacific Medical College & Hospitals approved the study.

Study setting

The sample collection and analysis procedures were conducted at Pacific medical college and hospital Udaipur Rajasthan, ensuring standardized protocols and quality assurance measures for This prospective observational study. Udaipur is having male population size of 1,566,801 according to the 2011 census. It is located at the coordinate of 24.5 degrees N and 73.6 degreesE. Also, popular as "City of Lakes".

Questionnaire

Study indicators include the participant's age, history of chronic disease, socio-economic status, geographical location, educational status etc. Trained residents who were posted at respective OPDs collected the data from participants. Data was collected on the paper-based structured questionnaire (signed and verified by Principal Investigator) and later entered into Microsoft Excel format for statistical analysis.

Semen specimen collection

Semen specimens were obtained from masturbation process into a sterile plastic container after 3-5

days of sexual abstinence. The collected specimens were left to liquefy at 370C for 20 min as per WHO2010.

Semen analysis

Semen specimens were analyzed for semen volume, sperm concentration, motility and morphology within 1h of collection according to World Health Organization guidelines [18]. Briefly, motile spermatozoa percentage was evaluated by placing 10µl of semen specimen over apreheated glass slide and observing at 4000 magnification microscope according to WHO standard protocol [18].

Sperm viability was assessed using the Eosin/Nigrosin stain method. Briefly, 1:1 ratio of eosin (20µl of 1%) and Nigrosin (20µl of 10%) were added to 10µl of semen specimen. A smear was fabricated on a clean glass slide and after drying unstained (intact) and stained (anomalies in membrane) spermatozoa were counted under oil immersion microscope with 100X objective. Percentage of intact cells was used to calculate sperm viability in semen specimens. Sperm morphology in freeze thawed specimens was performed according to previously published literature using Shoor-stained semen smear method [18].

Total antioxidant capacity

The total antioxidant capacity (TAC) of the semen specimens was estimated according to the previously published literature using the TAC calorimetric kit (TAC-2513, Bio-Diagnostic) [19]. Briefly, 20µl of the semen specimen was added to 500 µL of the H2O2 followed by incubation at 370C for the time period of 10 min. Chromogen (500 µL) supplied in the kit was added to the incubation mixture and further incubation was done at 370C for 5 min. The absorbance at 505 nm was recorded for specimens and blank. TAC levels were calculated using formula Absorbance of Blank –Absorbance of specimen x 3.3 and presented as µM/mL.

DNA fragmentation index

1. The DFI (DNA Fragmentation Index) test and SCD (Sperm Chromatin Dispersion) test are both laboratory techniques used to assess sperm DNA integrity, which is important for male fertility. DFI Test (DNA Fragmentation Index): This test measures the percentage of sperm with fragmented DNA. High levels of DNA fragmentation can impair fertility and increase the risk of miscarriage. The DFI test typically involves staining sperm with a fluorescent dye that binds toDNA, then analyzing the sperm under a microscope to identify fragmented DNA. Bright field microscopy can be used for this purpose, where the fragmented DNA appears darker compared to intact DNA.

SCD Test (Sperm Chromatin Dispersion Test): This test evaluates sperm DNA fragmentation by measuring the ability of sperm chromatin to disperse in an acidic environment. Intact DNA will produce a characteristic halo around the sperm head, while fragmented DNA will not disperse properly and will lack a halo. The SCD test is also typically performed using bright field microscopy. Tests provide valuable information about sperm DNA integrity, which is essential for successful fertilization and embryo development. They are often used in conjunction with other semen analysis parameters to assess male fertility potential comprehensively. The DNA Fragmentation Index (DFI) is a measure of the percentage of sperm with fragmented DNA. The formula to calculate DFI for each individual sperm can vary depending on the specific method used in the laboratory. However, a common method involves staining sperm with a fluorescent dye that binds to DNA, then analyzing the sperm under a microscope to identify fragmented DNA. One common formula used to calculate DFI involves counting the number of sperm with fragmented DNA (stained darker due to fragmentation) and dividing it by the total number of sperm counted. This yields a percentage representing the DFI for that sample [20].

Mathematically, the formula can be represented as:

$$\mathbf{DF�} = \frac{\textbf{NumberofSp�r�wit�Fragm�nt�dDN�}}{\textbf{TotalNumberofSp�r�Counted}} * 100$$

This formula provides the percentage of sperm with fragmented DNA in the sample, which is theDFI value for that sample. This value helps assess sperm DNA integrity and can be indicative of male fertility potential. These analyses were conducted at Pacific medical college and hospital Udaipur Rajasthan, employing standardized protocols and instrumentation.

Lipid Peroxidation

Lipid peroxidation level of semen specimens were assessed through quantification of malondialdehyde according to the previously published protocol using calorimetric assay kits [21]. Briefly, 1 mL of the chromogen supplied in the kit was added to the 200 µl of the semenspecimen followed by boiling for the period of 30 min. The color developed after cooling the specimen tube, was recorded as absorbance at 534 nm. Distilled water was used as blank during the experiment. The calculation of MDA level was performed using the formula (Absorbance of sample/A of standard standard × 10) and was presented as micromoles of MDA per sperm concentration.

Semen cryopreservation

Post semen analysis, each semen sample was cryopreserved according to the standard protocol previously mentioned in literatures using the spermfreeze[22]. Briefly, semen samples were diluted in the ratio of 1:0.7 using freezing medium added drop-wise till attainment of the equilibrium at room temperature for a time period of 10 min. The mixture was then immediately poured into the cryovials and then further stored in liquid nitrogen (-1960C). Semen specimens were cryopreserved for a time span of 7 days and then, thawed at room temperature for 20 min for further analysis as done before cryopreserving each specimen.

Statistical analysis

Statistical analysis was performed using SPSS software version 18. Non-parametric analysis was performed using SPSS software to compare the semen characteristics, TAC, DFI and malondialdehyde in pre and post cryopreserved semen specimens. The test was considered statistically significant when $p<0.05$. The relationship between semen parameters, DNA fragmentation, TAC and malondialdehyde was analyzed using Spearman's correlation coefficients.

Results

The results of the present study demonstrated that the mean±SD age of the study participantswere found be 34±1.8 years. The mean length of sexual abstinence was found to be 3.2 days witha range of 0-7 days. Among the study participants, twelve showed sexual abstinence for less than the recommended days prior to semen specimen collection and therefore excluded from the present study. The baseline characteristics of the semen analysis according to the WHO criteria and all parameters are shown in Table 1. The results of semen characteristics of both fresh and cryopreserved specimens were illustrated in table 1. The results showed there is significant decrease in means values of total sperm count (38.17±12.68 v/s 31.22±15.41; p 0.0006), totalmotility (39.37±6.60 v/s 34.66±8.19; p 0.0001), PR (24.00±6.7 v/s 21.42±2.84; p 0.0005),morphology (5.32±0.95 v/s 4.57+0.58; p 0.0001) and DNA fragmentation index (16.60±2.48 v/s24.79±4.49; p 0.0001) in cryopreserved specimens compared to fresh semen.

Table1:SEMENANALYSIS VARIABLESOFFRESH AND CRYOP RESERVED SPECIMENS

Semen characteristics	Fresh		Frozen		P value	CI at 95%
	Mean	SD	Mean	SD		
Total Count	38.17	12.68	31.22	15.41	0.0006	3.0146 to 10.8854
Total motility	39.37	6.60	34.66	8.19	0.0001	2.6358 to 6.7842
PR	24.00	6.7	21.42	2.84	0.0005	1.1450 to 4.0150
Morphology	5.32	0.95	4.57	0.58	0.0001	0.5305 to 0.9695
DNAfragmentation index	16.60	2.48	24.79	4.49	0.0001	-9.2015 to -7.1785

Table2:SERUMBIOCHEMICALVARIABLESOFFRESHANDCRYOPRESERVEDSPECIMENS

Semen Biochemistry characteristics	Fresh		Frozen		P value	CI at 95%
	Mean	SD	Mean	SD		
Total AntioxidantCapacity (µM/mL)	1764.33	105.46	1438.12	64.09	0.0001	301.8739 to 350.5461
Malondialdehyde(nmol/mL)	0.66	0.05	1.173	0.181	0.0001	-0.55003 to -0.47597

Table3:PEARSON'SCORRELATIONOFCRYOPRESERVEDSEMENVARIABLESWITHAGE

		Age	DFI	MDA	TCA
Age	PearsonCorr.	1	0.08951	-0.00276	-0.03719
	p-value	--	0.37585	0.97827	0.71337
DFI	PearsonCorr.	0.08951	1	0.04282	0.0377
	p-value	0.37585	--	0.6723	0.70958
MDA	PearsonCorr.	-0.00276	0.04282	1	-0.09791
	p-value	0.97827	0.6723	--	0.33249
TCA	PearsonCorr.	-0.03719	0.0377	-0.09791	1
	p-value	0.71337	0.70958	0.33249	--
2-tailedtestofsignificanceisused					

Table4:PEARSON'SCORRELATIONOFFRESHSEMENDFIWITHMDALEVELS

		DFIFresh	MDAFresh
DFIFresh	PearsonCorr.	1	0.16963
	p-value	--	0.09155
MDAFresh	PearsonCorr.	0.16963	1
	p-value	0.09155	--
2-tailedtestofsignificanceisused			

Table5:PEARSON'SCORRELATIONOFCRYOPRESERVEDSEMENDFIWITHMDALEVELS

		DFICryopreserved	MDACryopreserved
DFICryopreserved	PearsonCorr.	1	0.04282
	p-value	--	0.6723
MDACryopreserved	PearsonCorr.	0.04282	1
	p-value	0.6723	--
2-tailedtestofsignificanceisused			

Moreover, semen biochemical analysis of the fresh and frozen specimens showed significant changes as illustrated in table 2. The total antioxidant capacity (TAC) showed significant decrease in cryopreserved specimens compared to fresh (1764.33±105.46 v/s 1438.12±64.09; p 1.1),while cryopreserved semen specimen showed more lipid peroxidation level(1.173±0.18)compared to fresh specimen (0.66±0.05;p 0.001).

The Spearman's correlation between the age and semen characteristics along with semen biochemical variables is shown in table 3. The results of the correlation analysis showed that participant age doesn't have any association between the DFI, TAC, and MDA as illustrated in table 3. Similarly, table 4 and 5 illustrated positive correlation of DFI with MDA levels in fresh and cryopreserved semen samples respectively.

The interquartile distribution of study participant's for DFI, TAC and MDA were shown in Fig 1, 2 and 3 respectively.

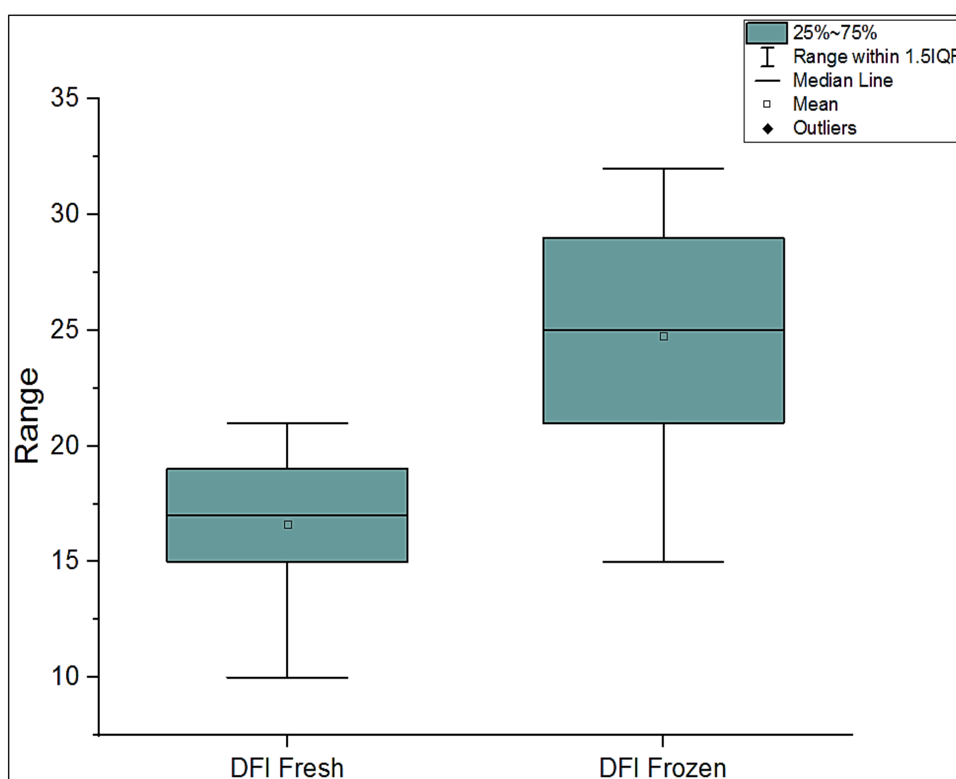

Fig 1:BOX PLOT OF SHOWING STUDY PARTICIPANT' SEMEN DFI IN FRESH AND FROZEN SPECIMENS

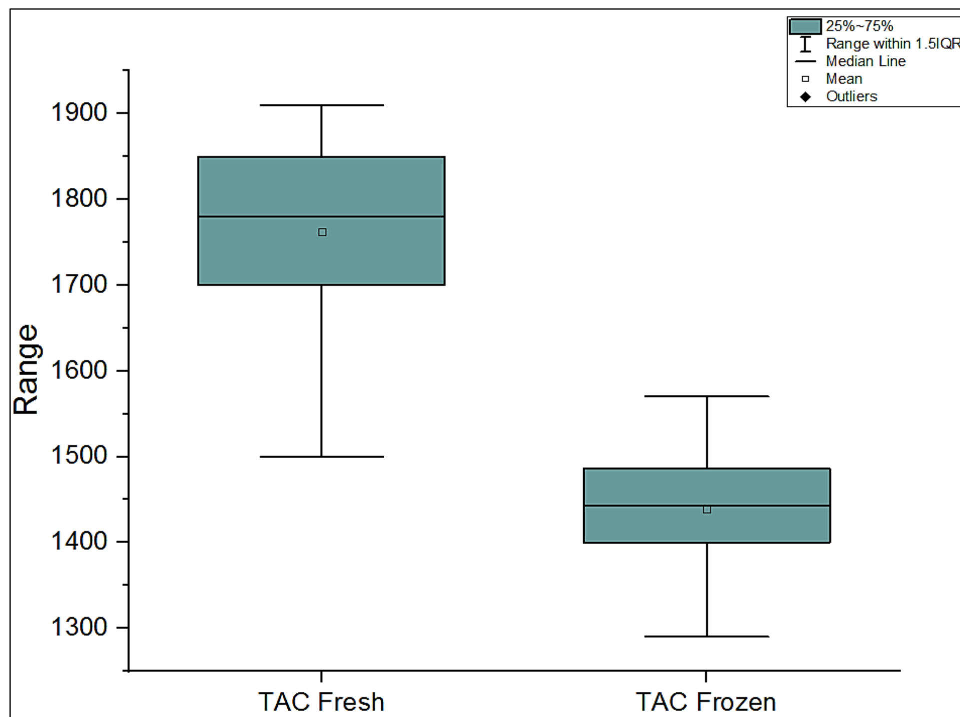

Fig 2:BOX PLOT OF SHOWING STUDY PARTICIPANT' SEMEN TAC IN FRESH AND FROZEN SPECIMENS

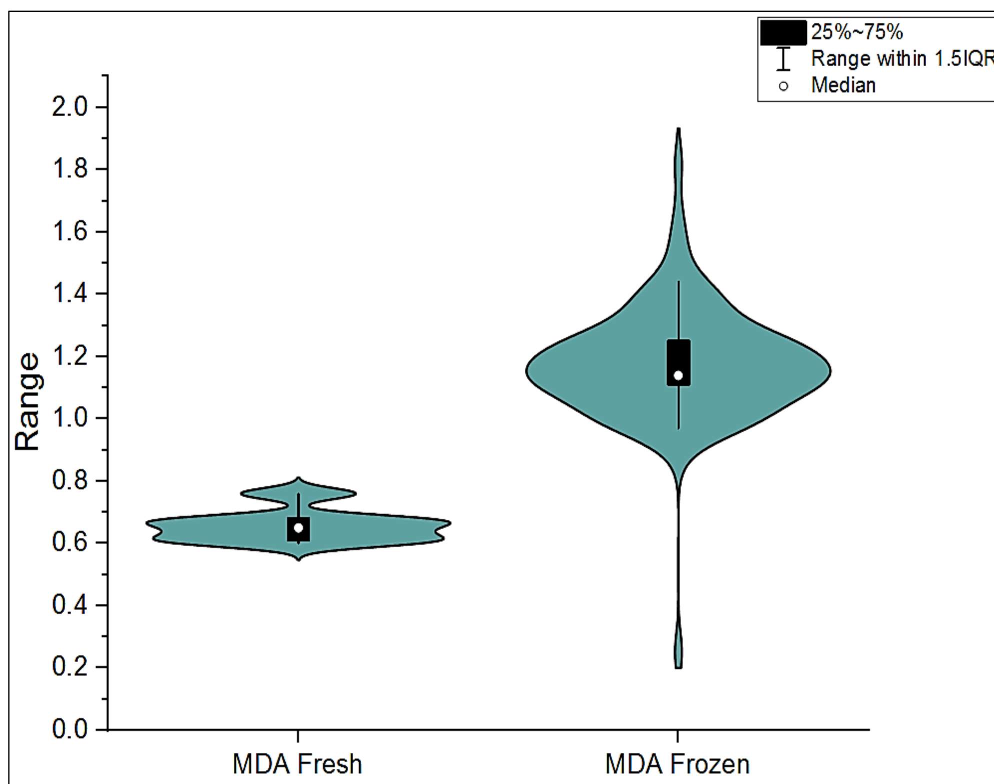

Fig 3:VIOLIN PLOT OF SHOWING STUDY PARTICIPANT' SEMEN MDA IN FRESH AND FROZEN SPECIMENS

DISCUSSION

The semen cryopreservation technique is an essential component of infertility management but somehow associated with limitations due to freezing and thawing events. This process of cryopreservation followed by thawing adversely affect the sperm morphology, mobility, viability, DNA integrity along with other biochemical parameters like MDA and TAC [23-24]. Previous studies also reported that cryopreservation of sperms cause's damages to the cells especially through the mechanism of reactive oxygen species (ROS) generation and lipid peroxidation mechanism of the membrane which can have negative impact on sperm fertility parameters [25-27]. In the present study we attempted to assess adverse effect of cryopreservation on semen characteristics and semen biochemical variables including total antioxidant capacity and malonaldehyde.

Cryopreservation and post thawing process induces the apoptosis cascade that is responsible for the sperm DNA damage [28]. Previously published study proved the role of caspase 3 in the initiation of apoptosis that further lead to DNA damage and fragmentation [29]. In our study we reported significant increased DNA fragmentation in the cryopreserved semen specimens after thawing as our findings are in complete agreement with the findings of previous studies [28-29].In our study, positive correlation between MDA, a lipid peroxidation marker and DFI was observed. This may be contributed due to formation of ROS in thawed samples due to activationof caspase 3 as evident from previous study [29]. Another study reported that freeze thawing generate the oxidative stress that also contribute to DNA damage of sperm cells and leas to increase in DFI accompanied with apoptosis [30].

Our results illustrated that there is increase in TAC and MDA activity in cryopreserved semen specimens compared to fresh semen samples. Seminal plasma is a rich source of antioxidants that protect spermatozoa from oxidative damages. Thawing process of cryopreserved semen specimens renders induction of oxidants and thereby affects the total antioxidant capacity and MDA levels. Sperm cell membranes are susceptible to peroxidative damage and MDA and therefore, these oxidative stress players contribute to generation of ROS within the seminal plasma [31].

Increased lipid peroxidation of spermatozoa in post cryopreserved thawed semen specimen has been credited with significant decrease in antioxidant enzymes resulting in reduction of total antioxidant capacity. Our findings also demonstrated reduction in TAC in cryopreserved specimen and are also supported by previously published study [32-33]. As a resultant of reduction in TAC within the cryopreserved samples, the spermatozoa specimen showed faster rate of peroxidation compared to fresh specimen [34].

Malondialdehyde concentration in seminal plasma contributes in determining the sperm viability, motility, morphology and volume [35-37]. Our study demonstrated positive correlation between MDA and DFI in both fresh and frozen semen samples, on contrary some of the few studies demonstrated no correlation among these two characteristics [38-39]. Since, considering the findings of our study, it is now clear that cryopreservation severely affect the biochemical and DFI characteristics of semen specimen.

Conclusion

In conclusion, our findings confirmed that cryopreservation followed by thaw mechanism of the semen specimens leads to devastating alterations in biochemical profile of semen plasma including total antioxidant capacity alongwith malondialdehyde. Moreover, due to generation of oxidative stress attributed by apoptosis also contributed towards DNA fragmentation in cryopreserved/thawed specimens. Still more interventions are needed to protect the spermatozoa cells from such harmful effect and some alternative methods should be developed for preservation of semen samples.

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