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Original research article

Association of DNA fragmentation index (DFI) and antioxidant enzymes as measure of sperm quality and fertility: 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 association between DNA fragmentation index and antioxidant enzyme.

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 to deleterious 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

Infertility is now among a common health issue that affects 8-12% of the couples of the reproductive age worldwide and among this, the male counterpart contribute around 50% of the cases [1]. Several papers have quoted the association between oxidative stress and pathophysiology of male infertility [2-3]. It has been observed that 30-80% of the males with infertility have significantly high levels of seminal reactive oxygen species (ROS) [4]. Under homeostasis, ROS involved as regulatory molecules in controlling the sperm functions such as capacitation, acrosome reaction, hyperactivation, and fertilization [5-6]. However, when the levels of ROS exceed the seminal antioxidant scavenging capacity, the oxidative stress occurred that can adversely affect the sperm quality and its cellular component [5-12].

The quality of sperm plays a pivotal role in fertility outcomes, making the assessment of various parameters crucial in understanding male reproductive health. Among these parameters, the DNA fragmentation index (DFI) and antioxidant enzymes have emerged as significant indicators, offering insights into sperm quality and potential fertility issues [6]. DFI measures the integrity of sperm DNA, reflecting the susceptibility of sperm to DNA damage. Sperm DNA damage can arise from various factors such as oxidative stress, environmental toxins, and abnormal sperm maturation processes [1-4]. High DFI levels are associated with reduced fertility outcomes, including lower rates of conception and higher risks of miscarriage. Therefore, assessing DFI provides valuable information on sperm quality beyond traditional semen analysis parameters. Antioxidant enzymes are critical in protecting sperm from oxidative stress-induced damage, which is a major cause of DNA fragmentation. These enzymes, including superoxide dismutase (SOD), catalase, and glutathione peroxidase, counteract reactive oxygen species (ROS) that can impair sperm function and DNA integrity. Their role is crucial in maintaining sperm viability and motility, thereby influencing fertility potential.

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Research has highlighted a direct relationship between DFI levels and the activity of antioxidant enzymes in sperm. Elevated ROS levels can overwhelm the antioxidant defense system, leading to increased DNA fragmentation. Conversely, deficiencies in antioxidant enzymes or excessive ROS production can compromise sperm quality by inducing DNA damage. Therefore, evaluating both DFI and antioxidant enzyme levels provides a comprehensive view of oxidative stress and its impact on male fertility. In clinical settings, measuring DFI alongside antioxidant enzyme activity assists in diagnosing male infertility and devising personalized treatment strategies. High DFI levels may prompt interventions aimed at reducing oxidative stress, such as lifestyle modifications (e.g., diet, exercise), antioxidant supplementation, or medical treatments targeting underlying causes of oxidative damage. Monitoring changes in DFI and antioxidant status during treatment allows for adjustments to optimize fertility outcomes.

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 methods

Study 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 degrees E. 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 37^0C for 20 min as per WHO 6^{th} edition.

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 a preheated glass slide and observing at 200/400 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 [12].

Routine examination

Briefly, 10 µL of the liquefied semen specimen was dropped at cell counting chamber followed by covering with the glass cover slip and analyzed. Minimum sperms were analyzed per specimen. Apart from this semen volume, semen concentration and sperm viability (as above) along with motile sperm ratio (PR) and non-forward motile sperm ratio (NP), and the immobile sperm (IM) ratios were calculated. Eosin-aniline black stain was used to determine the sperm survival rates. The analysis procedure was performed under the light microscope and out of 200 sperms and stained (dead) and living (unstained) sperms was counted for determining survival rates.

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Sperm Morphology
The semen specimens dried for 4h at glass slides along with PaP smear. At least 200 spermatozoa using oil based microscope with 100 X objective lens according to WHO Laboratory Manual for the Examination and Processing of Human Semen (6th Edition). The percentage of the spermatozoa with a normal morphology was estimated (4% considered to be normal; while less than 4% considered to be abnormal).

Acrosome integrity rate
Pap staining method was used to determine the sperm acrosome integrity rate using previously described method mentioned as Menkved approach [17]. Briefly, this method includes the counting of size of the vacuoles present in the sperm head at least in 200 spermatozoa. The integrity rate of acrosome was then calculated.

Sperm plasma membrane integrity
The integrity of sperm plasma membrane was calculated using hypotonic swelling test to determine the membrane integrity or intactness. Briefly, 20 µL of the semen specimen were mixed with hypotonic solution containing hypotonic solution (fructose and citric acid; 200µL). The mixture was incubated for 30 min at room temperature followed by smearing on a glass slide. The total number of spermatozoa with curved tails was investigated under light microscope.

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) (13). Briefly, 20µl of the semen specimen was added to 500 µL of the H_2O_2 followed by incubation at 37 ºC 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 37 ºC 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 to DNA, 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 [14].

Mathematically, the formula can be represented as:

$$\text{DFI} = \frac{\text{Number of Sperm with Fragmented DNA}}{\text{Total Number of Sperm Counted}} * 100$$

This formula provides the percentage of sperm with fragmented DNA in the sample, which is the DFI 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

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according to the previously published protocol using calorimetric assay kits [15]. Briefly, 1 mL of the chromogen supplied in the kit was added to the 200 µl of the semen specimen 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 × 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 [16]. 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 (-196 ºC). 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 linear regression.

Results

In present study the mean $\pm$ SD age of the study participants were found be 35.26 ± 2.14 years. In our study the study participants were distributed and found that majority were in the age group of 30-35 years (86%) followed by 14% participants within the age of >36 years. The mean length of sexual abstinence was found to be 3.4 days with a range of 0.8 days. Around ten participants due sexual abstinence were excluded from the study. The results of the present study demonstrated that with the cryopreservation, the sperm motility was observed to decreased significantly compared to the fresh specimen ($p<0.01$). There were noticeable changes between the normal seminal plasma before and after cryopreservation and levels in semen were found to be decreased upon cryopreservation as shown in table 1($p<0.01$).

The bridge plot was made to present the age wise distribution of the semen characteristics of the semen analysis according to the WHO criteria and all parameters are shown in figure 2. The results of semen characteristics of both fresh and cryopreserved specimens were illustrated in figure 2. The results demonstrated that majority of the participants showed age range between 30-35 and contribute in determining the significance of the each semen characteristics.

Moreover, semen count, morphology, motility of the fresh and frozen specimens showed significant changes as illustrated in figure 3. The total antioxidant capacity (TAC) showed significant decrease in cryopreserved specimens compared to fresh ($p<0.0001$), while cryopreserved semen specimen showed more lipid peroxidation level compared to fresh specimen ($p<0.0001$) as shown from one way ANOVA statistical analysis.

Moreover, DNA fragmentation index of the fresh and frozen specimens showed significant increase in the frozen specimen compared to fresh specimen as illustrated in figure 4 ($p<0.0001$). Total antioxidant capacity showed decrease in the cryopreserved specimen compared to fresh one as illustrated in figure 5 ($p<0.0001$). The levels of MDA showed increased level in significant amount in frozen specimens compared to fresh semen specimen as shown in figure 6 ($p<0.0001$).

In our study we performed the cluster analysis as shown in table 2 and figure 7 to identify natural groupings or clusters within a dataset. We performed this analysis to focusing solely on discovering and describing structures and patterns in the data without distinguishing between dependent and independent variables. We observed that characteristics of each cluster including all semen and biochemical parameters based on the coherence and differences between the groups and found to significant for all clusters ($p<0.0001$).

In table 3 and figure 8 we performed the linear regression fit model among the study variables to know the association and correlation between them including DFI, TAC and MDA. The Pearson's values obtained from this regression showed non-significant association between them self. However the graphs shown in figure 8 demonstrated high linearity between two variables and hence clearly showing association among themselves.

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Table 1: Distribution of study participants based on their age

Characteristic		N	%
Age	30-35	84	84
	>36	16	16

Table 2: Cluster analysis of Fresh versus Frozen specimens (ANOVA; p<0.0001) to be significant)

	Cluster DF	Cluster SS	Error DF	Error SS	F Value	Prob>F
DFI Fresh	1	197.80888	100	6.0756	32.55791	<0.0001
DFI Frozen	1	407.05833	100	20.01128	20.34144	<0.0001
TAC Fresh	1	2719534.09061	100	10878.74982	249.9859	<0.0001
TAC Frozen	1	1870612.49744	100	4056.26613	461.16612	<0.0001
MDA Fresh	1	0.36658	100	0.00246	148.85086	<0.0001
MDA Frozen	1	0.97496	100	0.03246	30.03557	<0.0001

Table 3: Linear regression fit model of Fresh versus Frozen specimens (ANOVA; p<0.0001) to be significant)

Equation	y = a + b*x					
Plot	DFI Fresh	DFI Frozen	TAC Fresh	TAC Frozen	MDA Fresh	MDA Frozen
Weight	No Weighting					
Intercept	16.52144 ± 0.56547	21.60203 ± 0.91538	1791.09423 ± 38.5371	1472.25001 ± 29.56994	0.67228 ± 0.01541	1.11088 ± 0.04049
Slope	-7.78993E-4 ± 0.00952	0.05791 ± 0.01541	-0.86935 ± 0.64888	-0.91327 ± 0.49789	-4.02762E-4 ± 2.59553E-4	0.00102 ± 6.81686E-4
Residual Sum of Squares	805.31516	2110.31105	3740271.96322	2202146.59197	0.59845	4.12805
Pearson's r	-0.00818	0.3517	-0.13279	-0.18042	-0.15334	0.14837
R-Square (COD)	6.69341E-5	0.12369	0.01763	0.03255	0.02351	0.02201
Adj. R-Square	-0.00993	0.11493	0.00781	0.02288	0.01375	0.01223

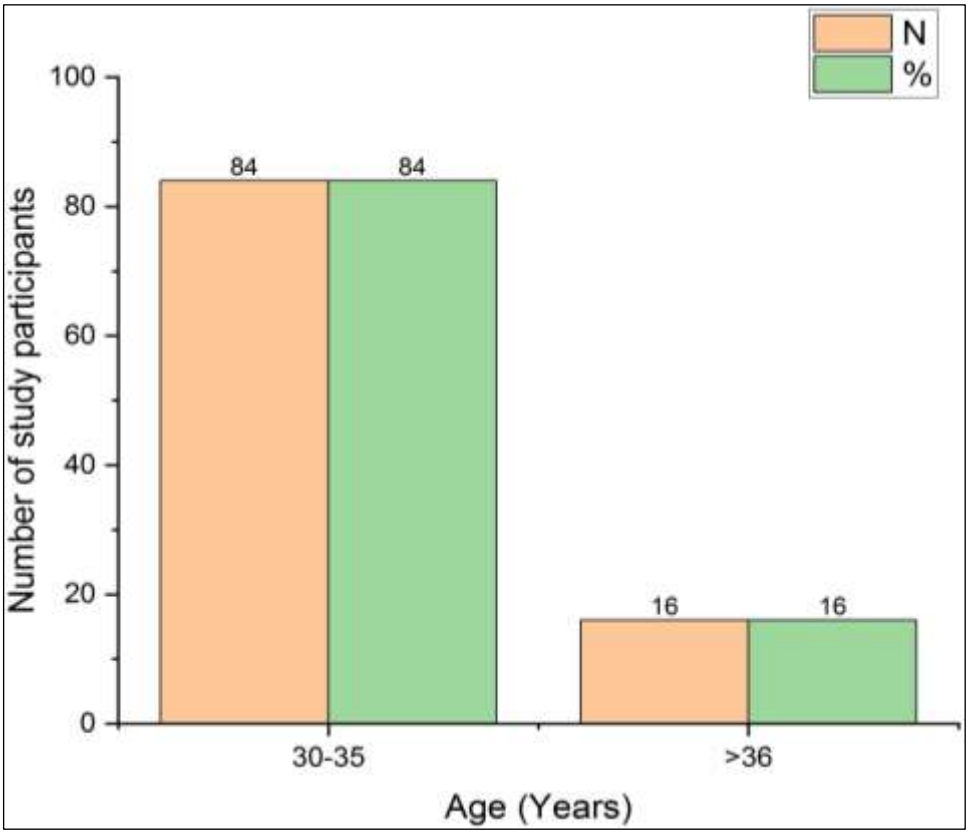

Fig 1: Histogram representation of age distribution of study participants

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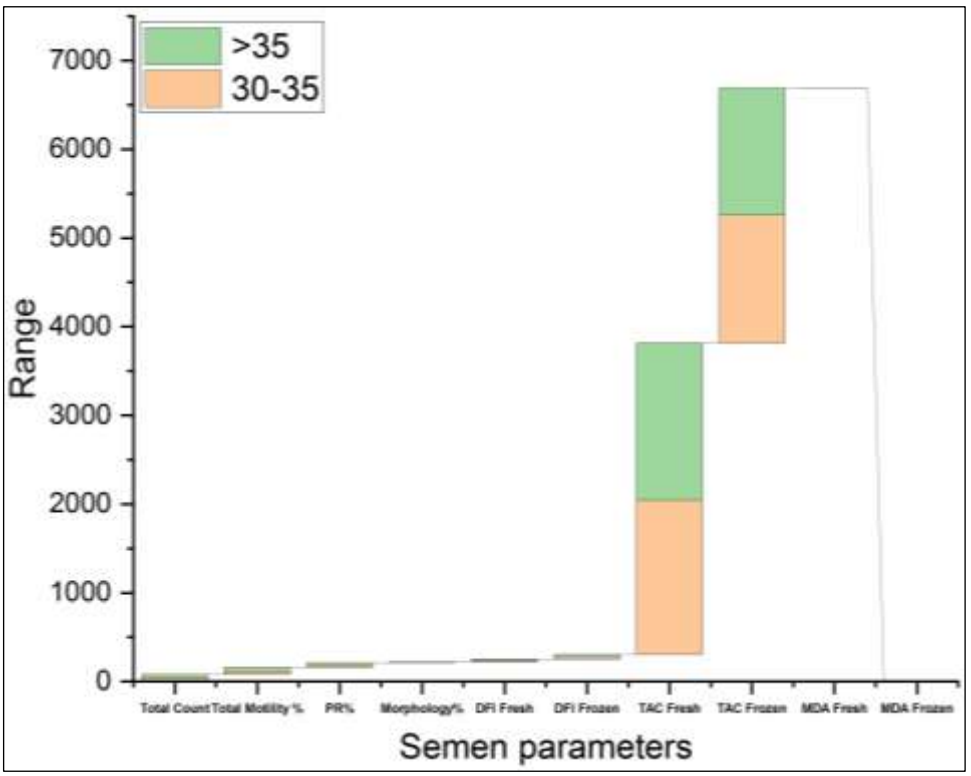

Fig 2: Bridge plot showing age wise distribution of semen analysis

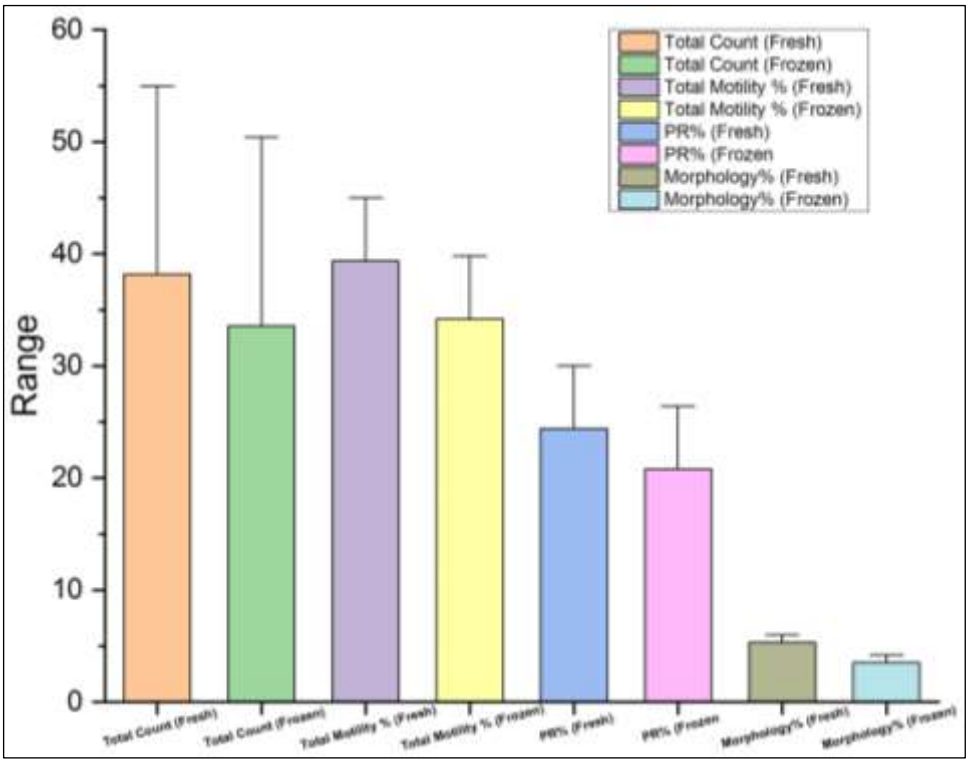

Fig 3: Comparative analysis of Fresh versus Frozen Semen specimen. (One way ANOVA ($p<0.0001$) to be significant)

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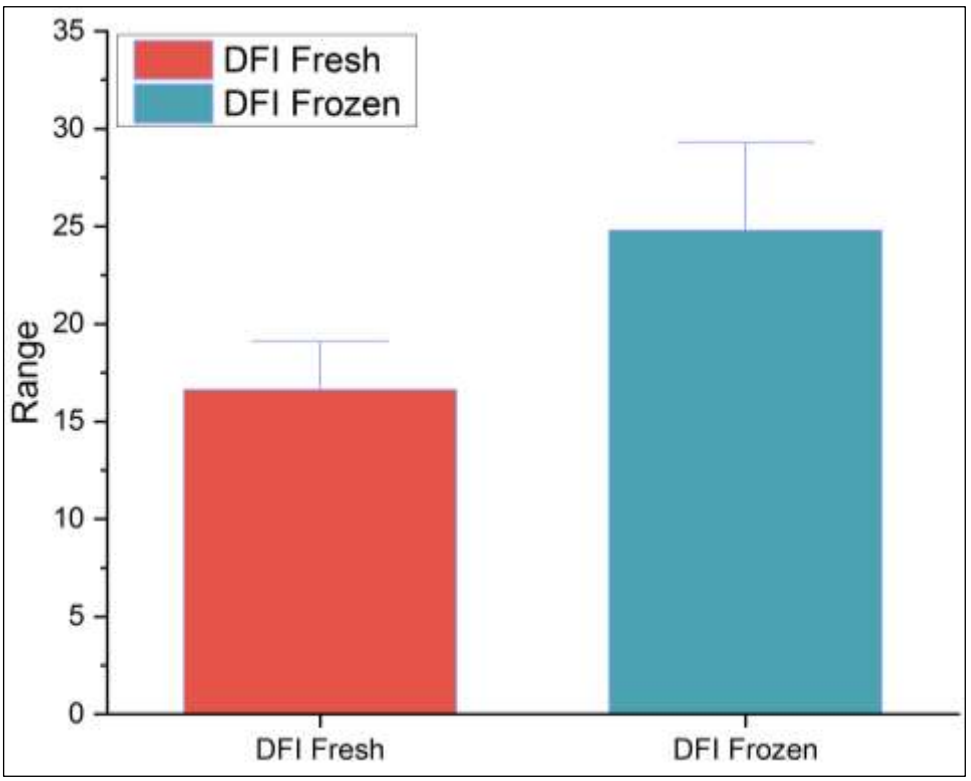

Fig 4: Comparative analysis of Fresh versus Frozen Semen specimen of DNA fragmentation index. (Student's "t" test ($p<0.0001$) to be significant)

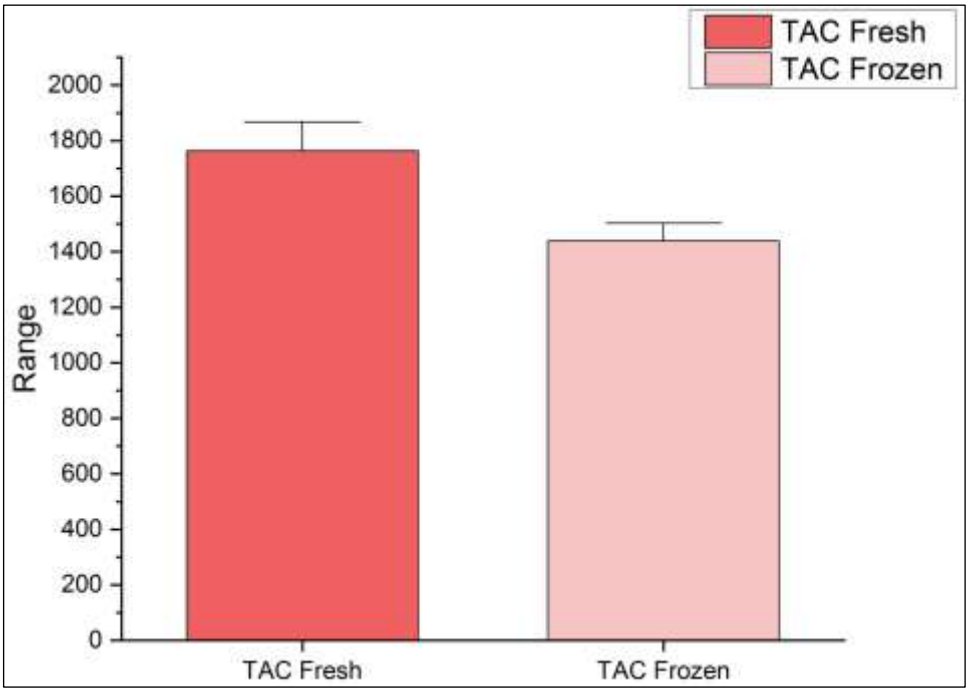

Fig 5: Comparative analysis of Fresh versus Frozen Semen specimen of Total Antioxidant Capacity (Student's "t" test ($p<0.0001$) to be significant)

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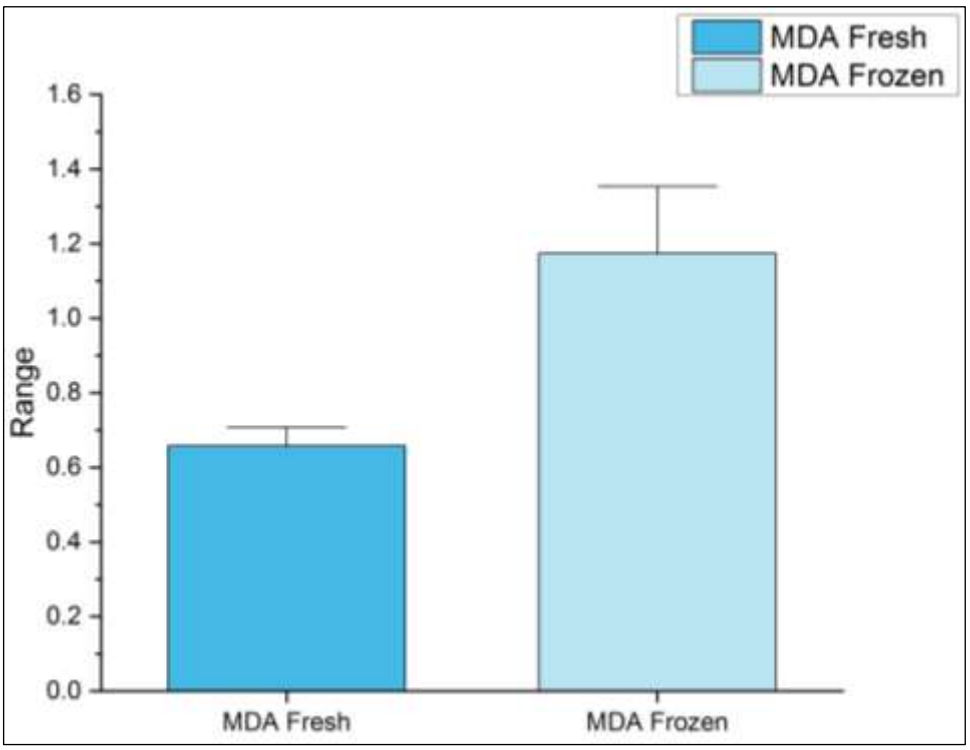

Fig 6: Comparative analysis of Fresh versus Frozen Semen specimen of MDA (Student's "t" test ($p<0.0001$) to be significant)

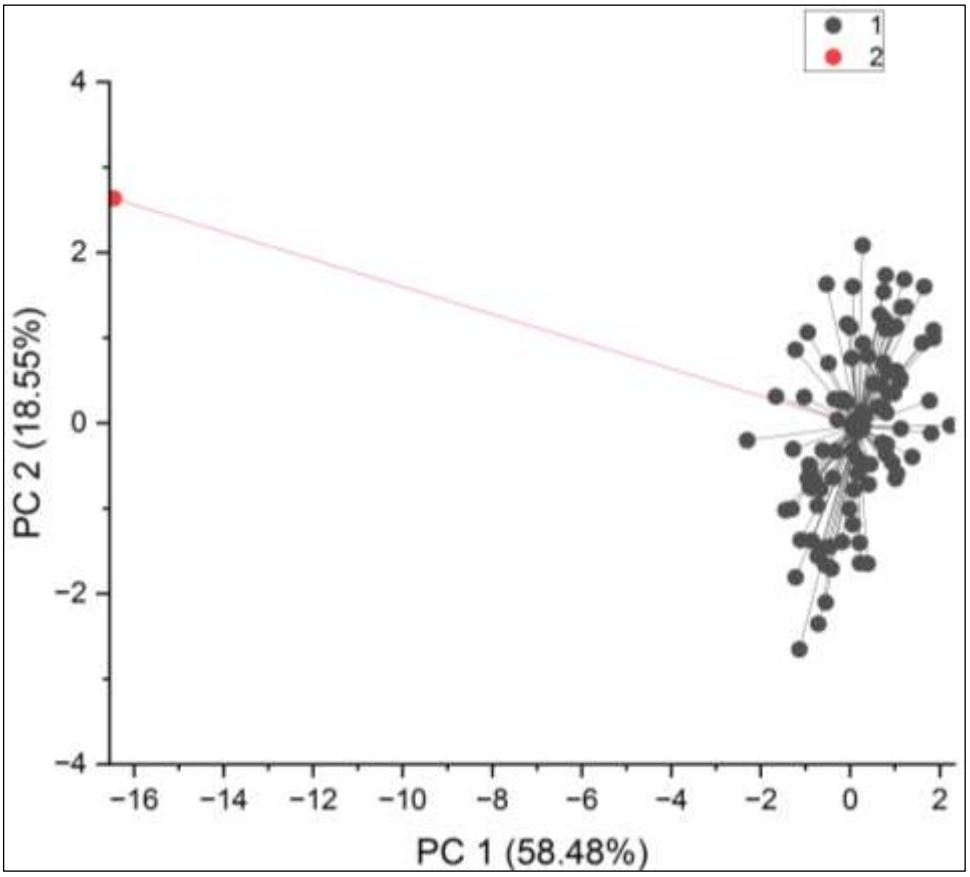

Fig 7: Cluster analysis of Fresh versus Frozen specimens (ANOVA; $p<0.0001$) to be significant)

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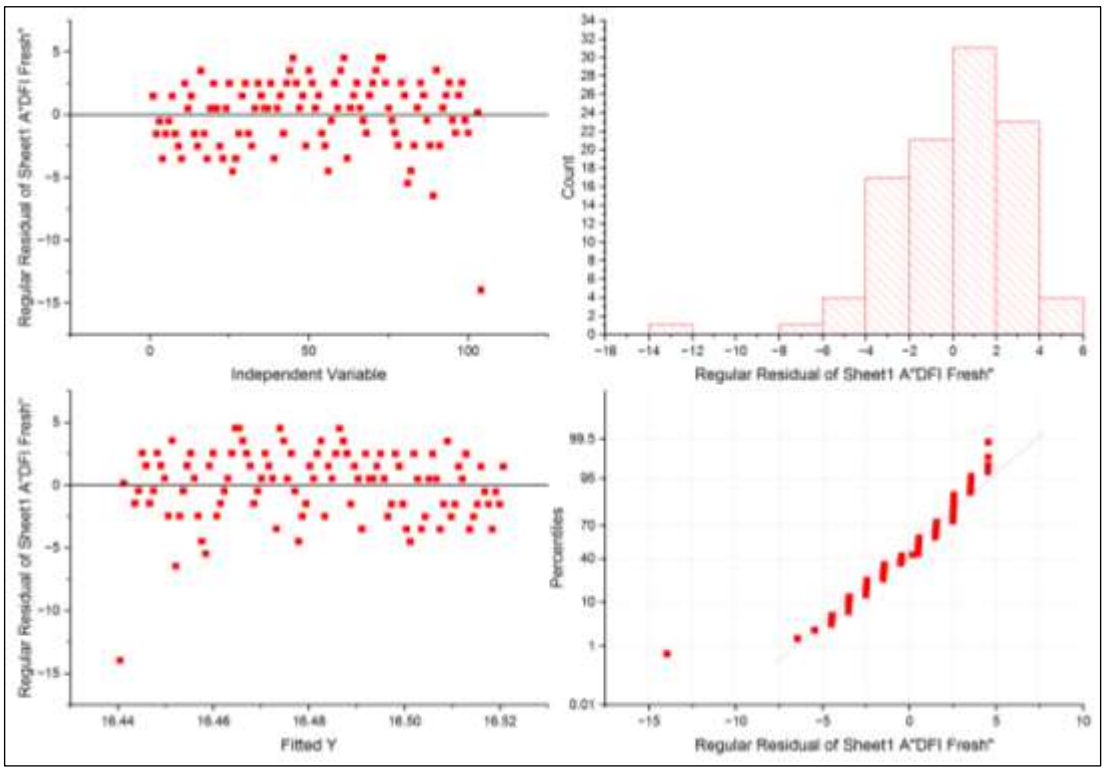
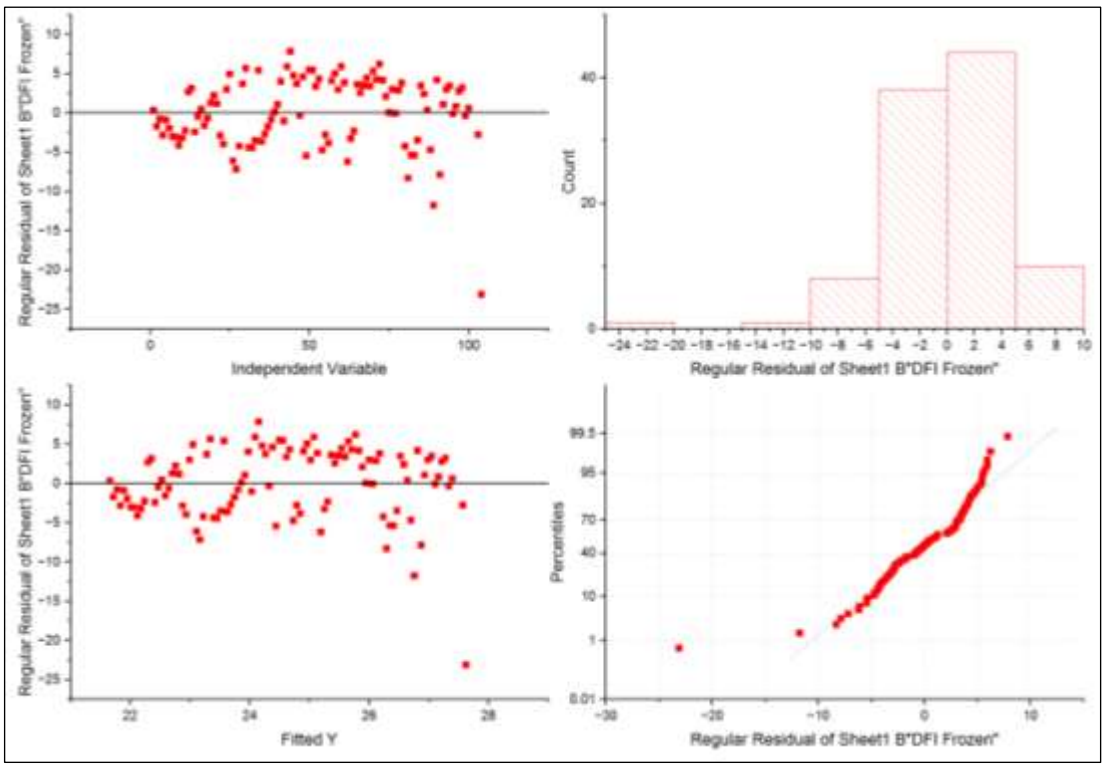


(A)



(B)

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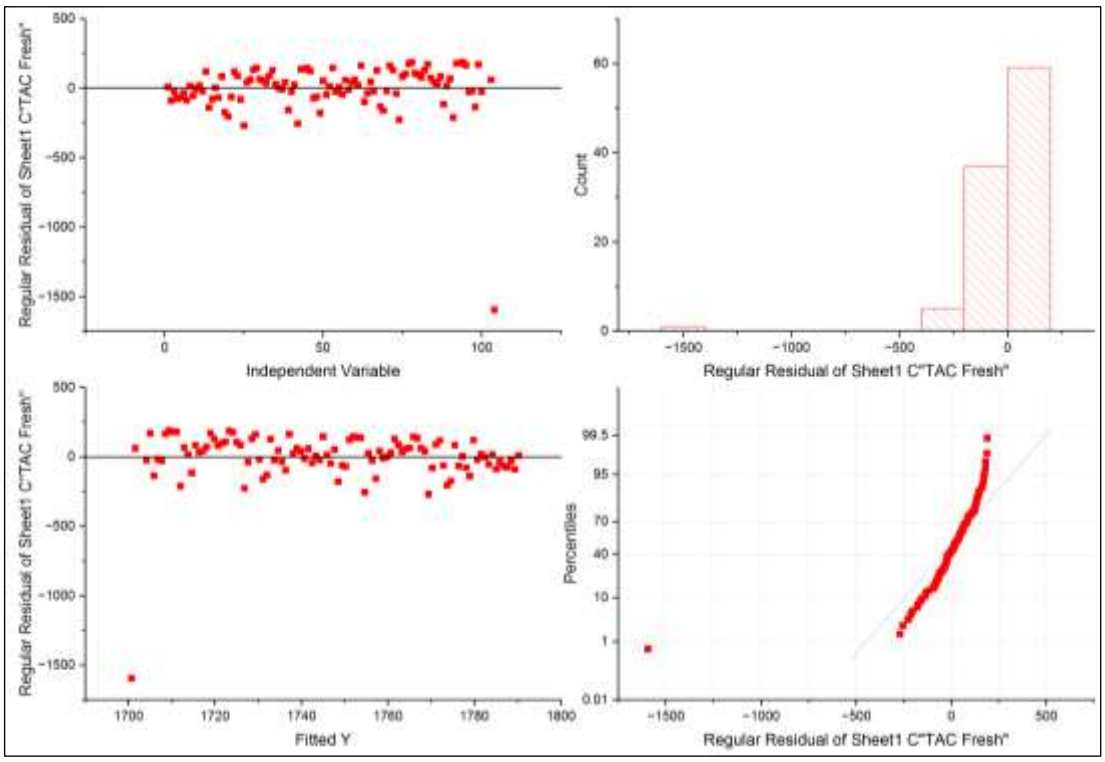

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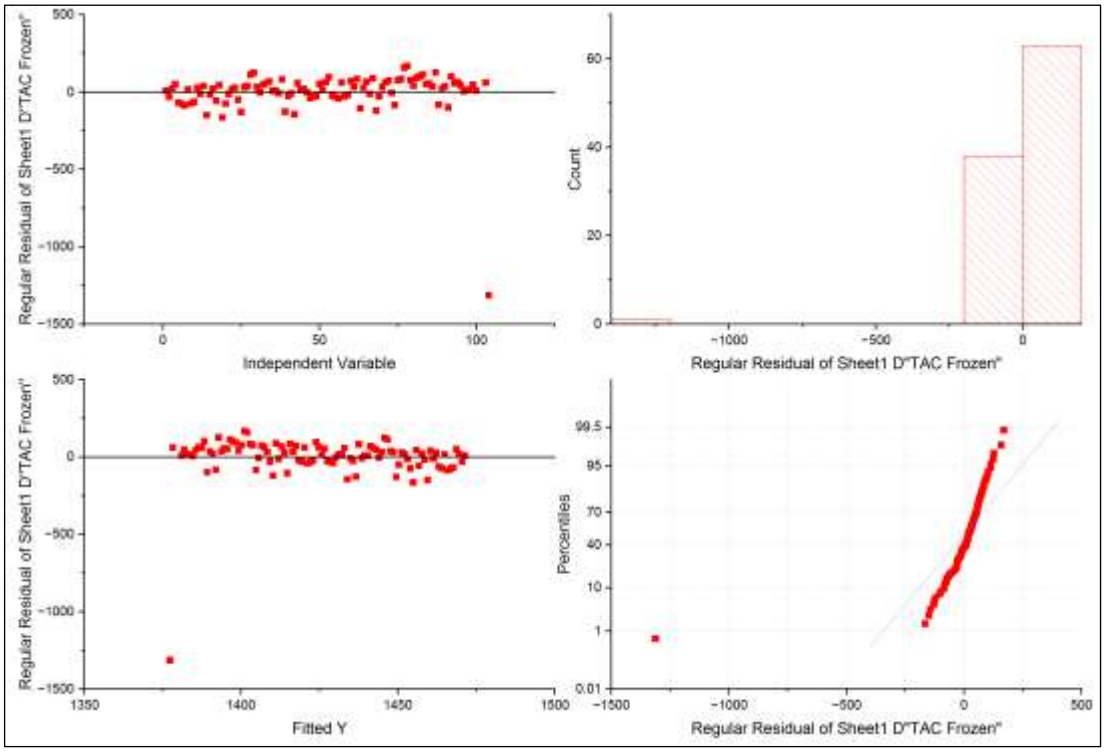

(D)

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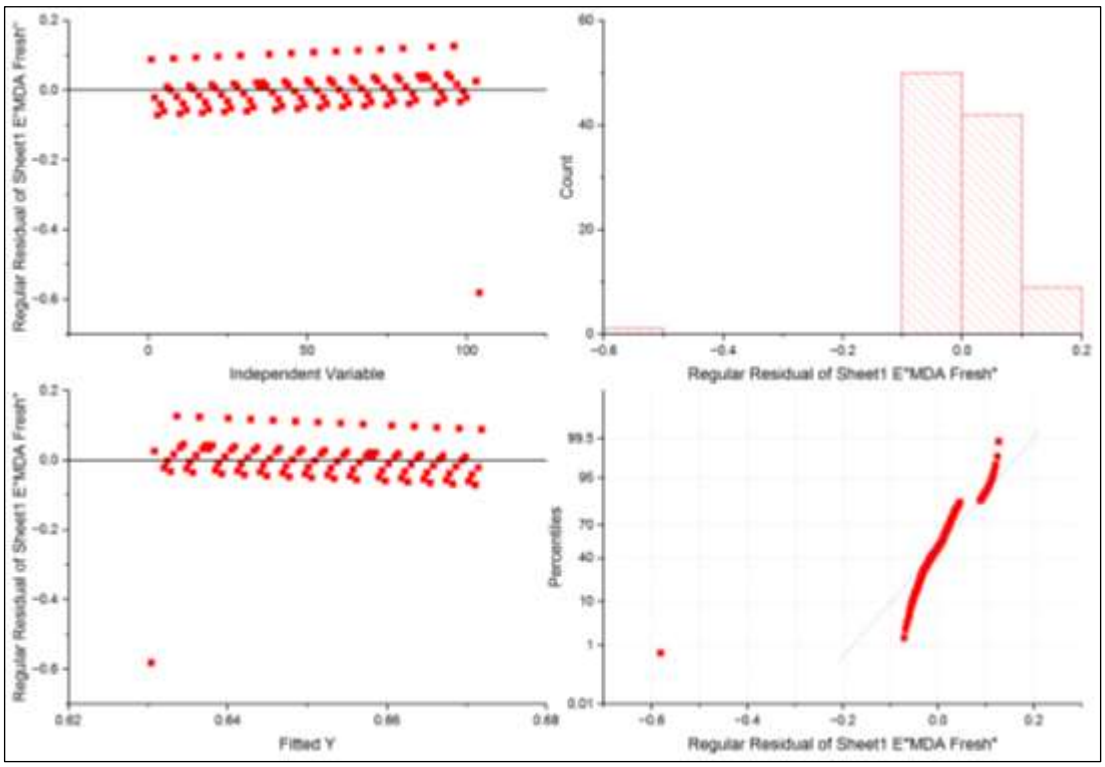
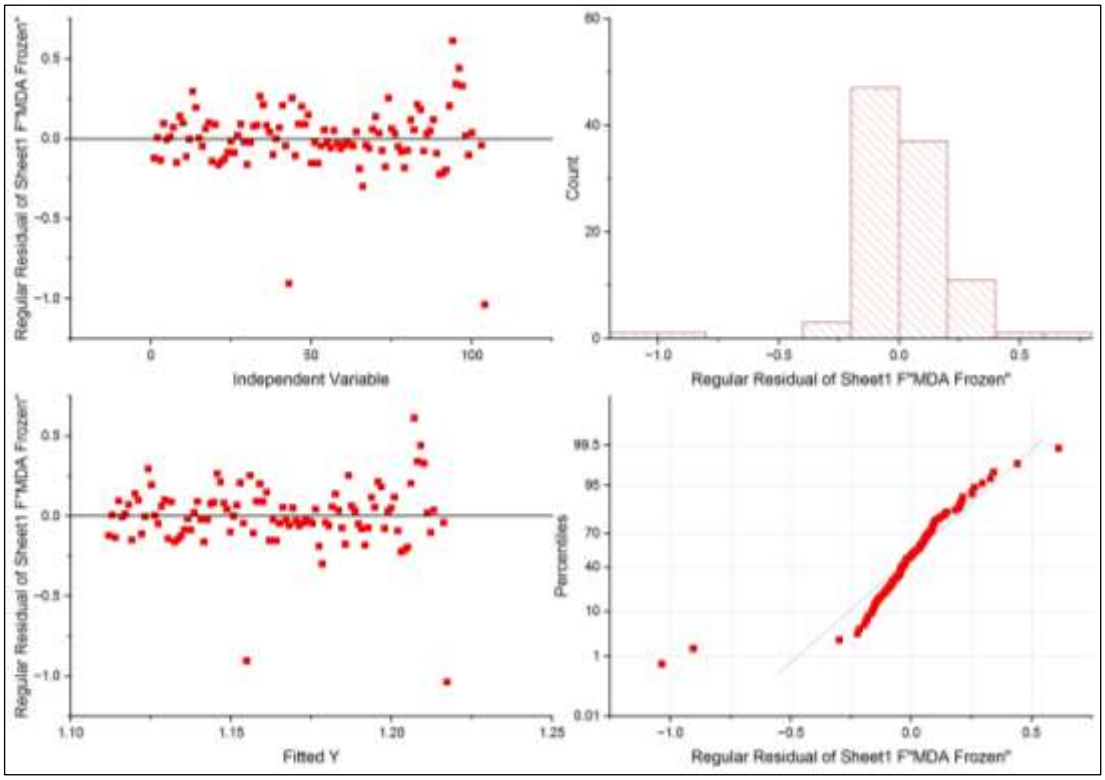


(E)



(F)

Fig 8: Linear regression fit curve of Fresh versus Frozen semen specimens (A) Fresh DFI (B) Frozen DFI (C) TAC Fresh (D) TAC Frozen (E) MDA Fresh (F) MDA Frozen

Discussion

Sperm cryopreservation approach is a classic example of assisted reproductive technology which has wide application in male population. In addition to the sperm cryopreservation for infertile males, it also helps in preserving and banking for specific male population having certain cancers in reproductive medicines [18]. The history of cryopreservation initiate in the year 1953 when Bunge and Sherman for the very first time developed a protocol for preservation of sperm in the presence of glycerol solution and dry ice. But this method was limited by the upto 67% of the sperm survival rate. Later, Sherman and

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their coworkers developed methods of human spermatozoa cryopreservation using liquid nitrogen [19].
The technique of semen cryopreservation is a vital part of infertility management, but it has limitations due to the processes of freezing and thawing. These processes can negatively affect sperm morphology, motility, viability, DNA integrity, and other biochemical parameters such as malondialdehyde (MDA) and total antioxidant capacity (TAC) (20-21). Previous studies have reported that cryopreservation damages sperm cells, primarily through mechanisms involving reactive oxygen species (ROS) generation and lipid peroxidation of the membrane, which can adversely impact sperm fertility parameters (22-24). In the present study, we aimed to assess the association between semen characteristics and biochemical variables, including total antioxidant capacity and malondialdehyde levels.
Our study also reported a significant increase in DNA fragmentation in cryopreserved semen specimens after thawing, aligning with the findings of earlier research (25-26). We observed a such changes in the levels of malondialdehyde (MDA), a marker of lipid peroxidation, and DNA fragmentation index (DFI), which may be due to the formation of reactive oxygen species (ROS) in thawed samples, possibly triggered by caspase 3 activation as suggested in previous studies (26). Another study indicated that the freeze-thaw process generates oxidative stress, contributing to sperm DNA damage and an increase in DFI associated with apoptosis [27]. Seminal plasma is rich in antioxidants that protect spermatozoa from oxidative damage. However, the thawing process of cryopreserved semen induces oxidant production, affecting both TAC and MDA levels. Sperm cell membranes are susceptible to peroxidative damage, with MDA and other oxidative stress markers contributing to ROS generation within the seminal plasma.
Increased lipid peroxidation in spermatozoa from cryopreserved-thawed semen has been linked to a significant decrease in antioxidant enzymes, leading to a reduction in TAC. Our findings also demonstrated a reduction in TAC in cryopreserved specimens, consistent with previously published studies [28-29]. This reduction in TAC results in a faster rate of peroxidation in spermatozoa compared to fresh specimens [30]. Malondialdehyde concentration in seminal plasma is associated with sperm viability, motility, morphology, and volume [31-33]. Our study demonstrated a positive correlation between MDA and DFI in both fresh and frozen semen samples, although some studies have reported no correlation between these two parameters [34-36]. Based on our findings, it is clear that cryopreservation significantly affects the biochemical properties and DFI of semen specimens.

Conclusion

In conclusion, our findings confirmed that the cryopreservation and subsequent thawing of semen specimens result in significant alterations in the biochemical profile of semen plasma, including total antioxidant capacity and malondialdehyde levels. Additionally, oxidative stress, attributed to apoptosis, contributed to DNA fragmentation in the cryopreserved/thawed specimens. Further interventions are needed to protect sperm cells from these harmful effects, and alternative methods for semen preservation should be developed.

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