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Effect of Novel Antioxidant L-Ergothioneine Supplementation To Human Semen Prior To Cryopreservation: Its Effect On Post Thaw Sperm Function And Chromatin Integrity

Yamini Asokan[1]*, Sanjeeva N Reddy[1]

1. *1.Sri Ramachandra University Sri Ramachandra Medical College Department of reproductive medicine and surgery Porur Chennai 600116, India*

ABSTRACT

The objective of the study was to assess the conservative effects of L-Ergothioneine (EGT), antioxidant, on the post thaw chromatin integrity of human spermatozoa. 400 normozoospermic semen samples were cryopreserved with (Test) and without (Control) supplementation of EGT. Clinically relevant tests such as Semen analysis (WHO 2010)[32], Sperm chromatin integrity (Sperm chromatin dispersion test) and Lipid peroxidation status (Malondialdehyde concentration assessment) were performed in both fresh and frozen thawed samples. It was seen that with the increase in ROS production due to cryopreservation, there is a significant decrease in the post thaw sperm parameters and sperm chromatin integrity (Fresh vs Control: $p<0.001$), the extent of which was reduced on addition of EGT (Test vs Control: $p<0.001$). Also, interestingly, the increasing ROS levels (assessed in MDA test) correlated directly with DNA damage. Hence the study provides a basal proof that EGT acts against the ROS and conserves the spermatozoa's functionality.

Keywords: Spermaozoa, Cryopreservation, Antioxidant

*Corresponding Author Email: yamini.asokan@gmail.com
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INTRODUCTION

The World Health Organisation (WHO) estimates the overall prevalence of infertility in India to be between 3.9 and 16.8 per cent (WHO 2004; Adamson *et al*., 2011)[1]. Assisted reproductive technology (ART), which includes intrauterine insemination (IUI), *in-vitro* fertilisation (IVF) and intra cytoplasmic sperm injection (ICSI), are used primarily for treatment of infertility.

With the increase in awareness and treatment availability, there is a rising demand for a suitable method of long-term storage of human reproductive cells. Due to the higher availability and easy attainability of spermatozoa, these cells were the first to be studied intensely in the field of reproductive cryobiology. With time, one of the most significant achievements in ART was development and use of sperm cryopreservation in cases of fertility preservation, convenience and donor banking (Cohen *et al*., 2012; Edgar & Gook 2013; Quas *et al*., 2013)[2,3,5]. Although sperm cryopreservation is an established technique, it is known to result in the reduction in motility and morphology (Nallella *et al*., 2004)[6], deteriorated viability (O'Connell et al, 2002)[4], damage to plasma membrane structure and function (Andreea *et al*., 2010)[7], DNA damage (Donnelly *et al*., 2001; Chohan *et al*., 2004)[8,9], apoptosis in frozen thawed spermatozoa (Paasch *et al*., 2004)[10] and hence negative effect on embryo quality and probability of blastocyst formation (Braga *et al*., 2015)[11]

The mechanisms behind the cryodamage to spermatozoa are thought to be multi-factorial; the excessive generation of reactive oxygen species (ROS) generated during cryopreservation has been suggested as a major contributing factor (Anger *et al*, 2003; Gadea *et al*., 2004)[12,13]. Accordingly, a variety of cryoprotective media, most supplemented with antioxidants, have been employed to overcome cryodamage (Yoshimoto *et al*, 2008; Li *et al*., 2010)[14,15]. Antioxidant supplementation has been shown to yield significantly improved quality of cryopreserved spermatozoa in experiments (Grossfeld *et al*, 2008)[16]. Hence, in this study, the structural and functional damage (evaluation of motility, viability, morphology and sperm chromatin integrity) caused due to cryopreservation was analysed and the simultaneous assessment of the effect of a novel antioxidant supplementation (L-Ergothioneine) to the cryo media on the post thaw spermatozoa was done. This would be a preliminary study which assesses the use of L- Ergothionine (LE) in human sperm study.

L- Ergothioneine (Betaine of 2-thio-histidine; EGT) is a unique naturally occurring antioxidant that is abundant in most plants and animals. Unlike the other available antioxidants those are used in sperm structural conservation, in physiological pH, it exists in thione form, hence have properties of lower redox potential (Does not undergo auto-

oxidation), slow degeneration rate (Stays for 4 days at RT in culture media), water soluble (less toxic than cysteine and glutathione) and serves as an antioxidant both *invivo* and *invitro*. Keeping this in mind, the objective of this study was to study the effect of L-Ergothioneine (EGT) supplementation to semen samples prior to freezing on its ability to maintain sperm lipid membrane constitution and thereby preserve the sperm chromatin integrity. Following which effect of ROS level (as determined by MDA in sample) was correlated to the extent of chromatin damage. Thus, determining if EGT could be used as an additive in day today use cryoprotectant media and thereby provide higher sperm retrieval post thaw.

MATERIALS AND METHOD

Chemicals used

The chemicals used for the study were all of analytical grade. The antioxidant supplement L-Ergothioneine was obtained from Biogenuix Medsystems Pvt. Ltd (New Delhi, India). The other chemicals used for the in-house preparation of media, buffer and solutions were procured from FertiPro (Belgium), Sisco Research Lab Pvt Ltd (Mumbai, India), Merck Specialties Pvt Ltd (Mumbai, India) and HiMedia (Mumbai, India).

Sperm Preparation

In the present study, 400 semen samples from men attending Department of Reproductive Medicine and Surgery, Sri Ramachandra University for infertility treatment were recruited. The average age of the male patients recruited for this study was 35 ± 4.9 years (range 23-48). The collection method was masturbation after a sexual abstinence of 3-5 days. After the semen was allowed to liquefy at room temperature, routine semen analysis (according toWHO2010) was done and the left-over samples proven to be normozoospermic were used for the study. As this is a preliminary study, only normozoospermic samples were used to first assess the L-EGT on sperm with normal functionality.

Sample allocation

The sample is split into three equal fractions. The first fraction is analysed for pre-freeze sperm qualitative and quantitative parameters. The remaining two parts are frozen in cryoprotectant with (Fresh) and without (Control) supplementation of L-Ergothioneine (EGT). The frozen sample is then qualitatively and quantitatively analysed post thaw to measure the effect of pre-freeze supplementation of novel antioxidant EGT.

Quantitative Analysis: Volume, Count, Motility, Morphology, Viability

Qualitative Analysis: Sperm Chromatin Integrity

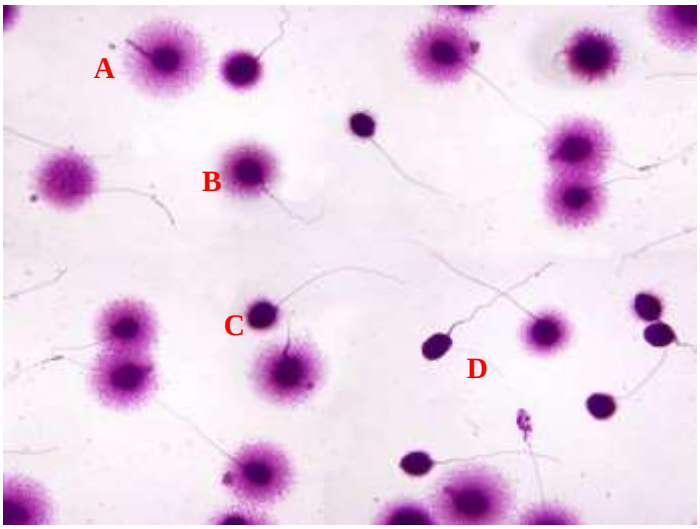

Figure I: DFI (%) in sperm- Sperm Chromatin Dispersion Test

The sperm which have intact chromatin exhibit Large Halo (A) and Medium Halo (B) on exposing it to the assay. The DNA fragmentation Index (DFI) is quantified by calculating the sum of spermatozoa which display Small Halo (C) and No Halo (D) around the head when stained in Giemsa and viewed under light microscope.

Sperm Cryoprotectant preparation with/ without supplement

The in-house preparation of Glycerol Egg-Yolk Citrate (GEYC) media was done as mentioned in WHO laboratory manual for the Examination and processing of human semen (5th Edition; 2010).

Sperm cryopreservation

Two fractions of the semen was cryopreserved in media with/without EGT by gentle mixing in the ratio 1:1 (Semen: cryomedia) - Control (semen + cryomedia) & Test (semen + cryomedia supplemented with 2mM EGT). The mixture was then transferred to a sterile cryovial (Nunc, Denmark) and plunged and stored in liquid nitrogen container (-196°C).

Determination of optimum EGT concentration

The dose optimisation was performed in 25 Normozoospermic semen samples. A stock solution (1M) of EGT was supplemented at different concentrations of 1.5mM, 2mM, 2.5mM, 3mM and 3.5mM and samples were stored in Liquid Nitrogen.

As shown in Table I, post-thaw study of semen samples supplemented with 2mM EGT had significantly higher percentage of functionality and DNA quality post thaw when compared to control ($p<0.05$). Therefore, 2mM of EGT was used for further studies.

Semen Analysis

All the assessment was done in duplicates and average was taken to minimize variations. A minimum of 5 fields per sample were evaluated, with a minimum of 200 spermatozoa counted per sample.

Concentration analysis was done by placing a drop (10µl) of sperm suspension on a Makler's counting chamber and the sperm density was expressed in millions/ml by observing under light microscope. The following tests were performed as per mentioned in the WHO2010 manual wherein the number of motile sperm was be graded and counted separately under light microscope (40X) and expressed in percentage. A thin smear of sperm suspension was prepared on a clean glass slide and stained by Diff Quick out of which percentage of sperm with normal morphology was counted. Sperm viability and membrane integrity was done using Hypo osmotic swelling test. The sample preparation was done by taking sperm suspension to swelling solution in the ratio 1:10 and incubating at 37ºC for 30 minutes and observed in magnification of 40X under light microscope. A total of 200 spermatozoa were tallied and the percentage of swollen spermatozoa was the measure number of intact membranes and hence the sperm's viability and membrane tension.

Hence the semen analysis was reported to confirm that the semen (N=400) used for the study was Normozoospermic according to the WHO 2010 lower reference range criteria (Table II)

Table I: Dose optimization of L- Ergothioneine (EGT) supplementation to human semen prior to cryopreservation

Groups (N=25)	Total Motility (%)	Prog Motility (%)	Capacitated (%)	Acrosome Rxtd (%)	MMP (%)	MDA (nmol/ml)
Fresh	51.56±8.99	38.6±8.72	38.84±9.14	29.36±9.99	66.32±2.12	0.973±0.17
Control	31.92±8.62	18.72±6.06	52.76±9.11	55.8±9.7	35.24±4.94	5.97±1.47
1.5mM	31.24±8.43	17.92±5.89	51.8±8.92	55.4±9.92	45.31±2.22	5.91±1.46
2mM	39.76±9.81*	26.12±8.30*	45±9.58*	50.96±9.46*	43.36±3.53*	3.84±1.43*
2.5mM	32.76±9.51	14.88±6.04	55.6±8.98	66.56±9.49	45.72±3.52	5.06±0.75
3mM	22.08±7.02	10.92±4.55	60.04±8.21	51.6±9.9	51.86±4.95	6.09±0.69
3.5mM	16.28±6.99	7.4±3.67	66.8±8.76	69.76±9.67	55.7±2.65	6.81±0.78

* $p<0.05$: Significant difference observed among all parameters: 2mM vs Control

Table II: Semen Analysis performed prior to use in study

Parameter (N=400)	Sub Classification	Values	WHO 2010 Lower Reference Limit
Volume		3±10.17	1.5 (1.4–1.7)
Ph		8.5	7.2
Vicosity		Normal	
Count (M/ml)		57.6±16.9	15 (12–16)
Motility (%)	Total	56.6±1.41	40 (38–42)
	Progressive	38.5±6.36	32 (31–34)
Morphology (%)	Normal	8±0.7	4 (3.0–4.0)
	Abnormal	91.9±0.7	
	Head	55.5±5.6	
	Neck	35.5±5.6	
	Tail	0.8±0.7	
Viability (%)		70.4±1.41	58 (55–63)

Measurement of lipid peroxidation (Malondialdehyde concentration; MDA):

MDA concentration, an index of lipid peroxidation, was measured according to the thio-barbituric acid reaction (Rao *et al*., 1989; Esterbauer *et al*.,1990)[17,18]. The semen fraction was removed and the sperm was resuspended in phosphate buffer saline. Equal volumes of diluted semen was mixed with cold 20% (w/v) trichloroacetic acid to precipitate the sperm proteins and pelleted. The final supernatant was incubated with equal volume of thiobarbituric acid reagent (0.67gm of thiobarbituric acid dissolved in 100 ml of distilled water with 0.5gm NaOH and 100ml glacial acetic acid). The suspension was heated for 1 hour in boiling water. After cooling, the supernatant was prepared and absorbance was read on the bio-spectrophotometer at 535nm.

DNA integrity analysis by Sperm Chromatin Dispersion (SCD) test:

SCD was performed as described by Fernandez *et al*., 2003 with minor modifications. The fresh and frozen thawed semen samples were diluted to get a concentration of 5 million/ml and mixed with equal volume of 1% LMPA (low melting point agarose). Approximately 150µl of the suspension was layered on slide pre-coated with 0.65% NMPA (Normal Melting Point agarose). The slides were placed in 4°C for 8 minutes to allow the agarose to solidify and produce a microgel with the sperm cells embedded within. The slides were immediately immersed in denaturation solution (0.08N HCl) in dark for 7 minutes. The slides were then subjected to lysis by placing them in lysing solution I (0.4M Tris HCl, 50 mM EDTA, 1% SDS, 20 mM DTT) for 10 minutes in RT followed by immersing in lysing solution II (0.4M Tris HCl, 2M NaCl) for 10 minutes in RT. After washing for 2 minutes with neutralising buffer (0.4M Tris HCl), the slides were then dehydrated in increasing concentrations of ethanol (70%, 90%, 100%) for 5, 5 and 10 minutes each respectively, air-dried and stained using Giemsa (working solution: 1ml of Giemsa stock with 8ml of DDW) and checked for DNA damage under light microscope. For each slide a minimum of 500 spermatozoa were scored. The spermatozoa were classified as follows:

Spermatozoa with chromatin damage: With no halo, small halo and fragmented

Spermatozoa with intact chromatin: With large and medium sized halo

Statistical analysis:

The data obtained were analysed with SPSS 21.0 version. To describe about the data, mean and standard deviation was used. The significant difference in the multivariate data analysis, one way ANOVA with Turkey's Post-Hoc test was used. To assess the relationship between the variables, Pearson's correlation with Scatter Plot was used. In all statistical tools, the probability value of $p<0.05$ was considered as significant value.

RESULTS AND DISCUSSION

Prior to sample inclusion, initial general characteristics of the 400 semen samples were assessed and confirmed to be Normozoospermic. As shown in Table II, all the semen samples had values more than the mentioned lower reference limit as mentioned in WHO manual, hence proving them to be normal and appropriate to be included for the study.

Table III: Comparison of post thaw sperm functional parameters in sperm supplemented with Ergothioneine

Parameter (N=400)	Fresh	Control (GEYC)	Test (GEYC+2mM)
Total Motility (%)	42±10.586	25.5±10.25*	30±9.97[a]
Progressive Motility (%)	36.5±7.86	18.5±6.71*	23±6.63 [a]
Non Progressive Motility (%)	5.5±9.09	7±5.11*	7±6.45
Viability (%)	66±9.35	39.5±10.4*	50±9.98 [a]
MDA (nm/ml)	1.08±0.14	5.94±1.06*	2.46±0.79 [a]
DFI (%)	30.05±8.94	41.7±11.45*	35.07±8.68 [a]

(*) $p<0.05$: Significant difference: Fresh vs Control

(a) $p<0.05$: Significant difference: Control vs Test

Cryopreservation induces regression of sperm Motility and Viability

The freeze thaw procedure protocol, even though is standardized, was observed to cause reduction in the overall quality of the sperm, thus making it suboptimal to use in certain clinical practices (Table III).

Progressive effects of supplementation of EGT in sperm cryopreservation media was observed with increase in post thaw quality

Different concentrations of EGT had varied effects on the sperm. This ascending trend of quality declined with all the concentrations above 2mM (2.5mM, 3mM and 3.5mM).

Effect on Motility and Viability:

It was observed that, in a semen split for analysis, there was an overall improvement in the post thaw quality of the semen fraction cryopreserved with supplementation when compared to the part which was frozen without it. The test group showed higher preservation ($p<0.05$) when compared to control groups of total motilities (Control vs 2mM: 25.5±10.25 vs 30±9.97), progressive motility (Control vs 2mM: 18.5±6.71 vs 23±6.63) and viability (Control vs 2mM: 39.5±10.4 vs 50±9.98).

Effect of lipid peroxidation:

The results for lipid peroxidation levels displayed in terms of Malondialdehyde concentration, indicative of oxidation status, after cryopreservation was displayed in Table III. The analysis of treatment effects showed that on EGT supplementation, there is lesser oxidation of sperm ($p<0.05$) when compared to control (Control vs 2mM: 5.94±1.06 vs 2.46±0.79).

Effects on sperm chromatin integrity:

SCD directly determines the extent of integrity in the sperm chromatin. As mentioned in Table III. The DFI (DNA Fragmentation Index), a combined value of number of sperm exhibiting No Halo and Small Halo (Figure I), was indicative of extent of sperm chromatin damage. There was a significant increase in cryopreservation induced DNA damage (Fresh vs Control: 30.05±8.94 vs 41.7±11.45) which was reduced significantly in Test group (Control vs Test: 41.7±11.45 vs 35.07±8.68).

Correlation between ROS levels (MDA ng/ml) with DNA Fragmentation Index (%)

It was observed that the increasing levels of ROS generated in the semen during cryopreservation had a significant correlation ($p<0.05$) with the increase in externally induced chromatin damage in sperm (Positive Correlation). Figure II shows a positive correlation with r= 0.127 (p=0.011*) on correlating the ROS generated with the DFI in sperm in the Control group. Figure III shows a positive correlation with r= 0.125 (p=0.012*) on correlating the ROS generated with the DFI in sperm in the Test group.

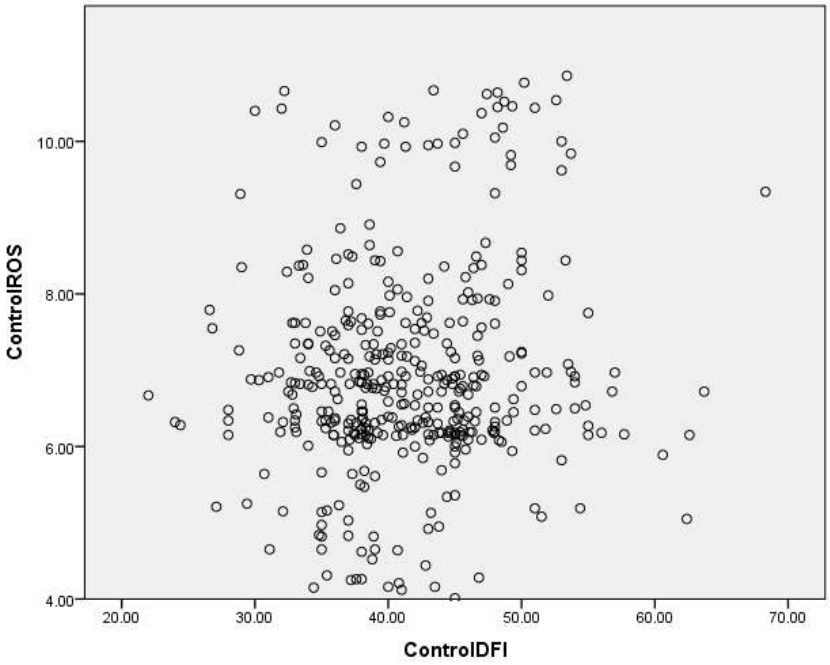

Figure II: Correlation of ROS levels (MDA ng/ml) DFI in Control group

Scatter plot formed on comparison of Control ROS with Control levels DFI. This gives the correlation of extent of influence of the ROS on the DNA damage in Fresh group.

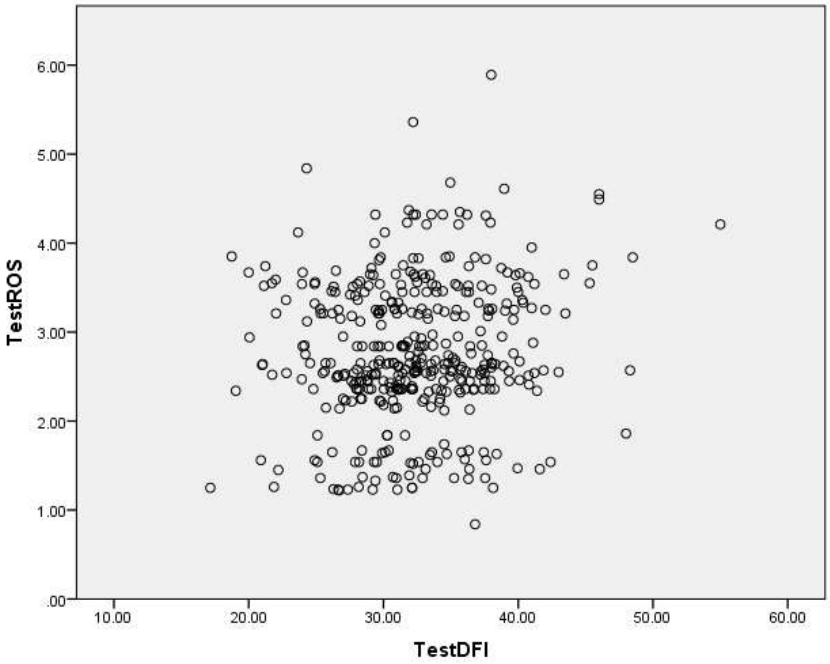

Figure III: Correlation of ROS levels (MDA ng/ml) DFI in Test group

Scatter plot formed on comparison of Control ROS with Control levels DFI. This gives the correlation of extent of influence of the ROS on the DNA damage in Test group.

DISCUSSION

Technique of human semen cryopreservation is an important technique routinely employed in the clinical management of male infertility (Medeiros *et al*., 2002)[19]. Sperm possess cellular organelles; the susceptibility of which to freeze–thawing damage may differ depending on accessibility to cryoprotectant (O'Connell *et al*., 2002)[4].

Motility is one of the parameters most seriously affected by freezing (Watson, 1995). It is also a strong predictor of the ability of a given sample to achieve fertilization in vitro (Donnelly et al., 1998). Despite its importance, the mechanism by which motility is reduced on cryopreservation has not been elucidated. Some studies report alteration in the DNA integrity of human sperm following cryopreservation (Thomson et al., 2009a; Zribi et al., 2010; Meamar et al., 2012)[20],. Men with spermatozoal DNA damage appears to have a decreased ability to father offspring and potentially have an adverse effect on embryo quality and spontaneous pregnancy loss. A recent study on marsupial sperm has elegantly demonstrated the importance of the osmotic changes in provoking chromatin relaxation and/or DNA fragmentation (Johnston et al., 2012

It is hypothesised that cryopreservation by itself is known to produce oxidative stress on the sperm, causing irreversible structural and functional damage to it thus reducing the fertilizing ability (Donnelly *et al*., 2001). This oxidative stress is attributed to the ROS (Reactive oxidative species) generated during cryopreservation and various studies have elucidated its

occurrence and effect on sperm (Peris *et al*., 2007; Thomson *et al*., 2009)[20,21].Even though the above mentioned negative correlation between ROS and IVF fertilization rate has been found (Zorn *et al*., 2003)[22], controlled generation of ROS has shown to be essential for the development of capacitation and hyperactivation; the two processes of sperm that are necessary to ensure fertilization. In vivo physiological concentrations of ROS are involved in providing membrane fluidity, maintaining the fertilizing ability and acrosome reaction of sperm (Agarwal *et al*., 2003)[23]. Hence it would be enough to control the externally produced excessive ROS, generated during cryopreservation, to prevent the detrimental effect of it on sperm quality. To facilitate this, supplementation of antioxidant to the cryo media has been a under study in various primate, mammal and human study (Pena *et al*., 2003; Michael *et al*., 2007; Chi *et al*., 2008; Taylor *et al*.,2009)[24-27].

In the present study, a novel antioxidant, EGT, was supplemented at different concentrations, to the sperm freeze media and the post thaw parameters were studied. This was a preliminary study that was conducted to conclude on a single concentration that would provide positive effects on the sperm's overall quality on thawing. This antioxidant, EGT, is a lesser explored chemical which serves as an antioxidant both *in-vivo* and *in-vitro*. The present study is the first to use EGT in human sperm preservation.

Preliminary experiments were conducted with concentrations of 1.5mM, 2mM, 2.5mM, 3mM and 3.5mM. The results of the study confirmed that the use of 2mM of EGT supplemented to the GEYC media showed higher preservation of sperm basic functional parameters and DNA integrity. This conservation could be a consequence of its antioxidant properties thus inhibiting formation of hydroxyl radical (Motohashi and Mori, 1986)[28], superoxide anions and singlet oxygen production (Obayashi *et al*., 2005)[29] at that particular measure.

Çoyan *et al* (2011)[30] studied the effects of adding different levels of EGT (1, 2 and 4 mM) to a ram semen extender containing egg yolk, and found the increasing levels of EGT led to positive effects on the total and progressive motility and several kinetic parameters. Likewise, in the present study, on comparison with the control, there was a higher conservation of total and progressive motility ($p<0.05$). This preservation could be due to the ability of EGT to conserve the mitochondrial membrane potential that in turn positively affects the motility parameters of the sperm.

Similarly, various studies have deliberated in detail and established the fact that the fertilization capacity of the human spermatozoa was found to be preserved on addition of antioxidant (Chi *et al*., 2008)[31]. The important functional aspect of the spermatozoa is its DNA which contributes to the paternal set of chromosomes on fertilization. On addition of EGT, it was seen that there was a reduction in the amount of spermatozoa with damage in

DNA. Thus, giving an insight into the fact that the generation of ROS could be a possible reason for the premature reaction and this is substantially controlled on the addition of the antioxidant pre freeze.

The membrane of the spermatozoa is the most prone to damage due to ROS generation due to the high PUFA and lipid content (Bucak *et al*., 2010). Hence as an indicator of membrane damage, malondialdehyde generated as an end product was assessed through the thiobarbituric acid assay. In the present study, ROS generated was accounted by the assessment of by-product, malondialdehyde, produced. Which was further correlated with various parameters of sperm under scrutiny. From the results, it was observed that the amount of ROS produced had significant correlations ($p<0.05$). Hence, the ROS generated is counteracted by the antioxidant added that henceforth conserves the membrane from undergoing damage and loss of viability.

CONCLUSION

In the present study, there was a significant conservation of sperm oxidative and qualitative functionality post thaw seen on supplementation of EGT at 2mM to the cryomedia. This is a preliminary study to assess the primary effect of EGT, the antioxidant, on spermatozoa. On account of strong correlation observed throughout the study between the parameters and ROS levels, it is concluded that the regressive action of ROS on the sperm was substantially corrected on addition of EGT. Thus, the antioxidant EGT could be used as a suitable additive for cryopreservation of spermatozoa.

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