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Conservative Effect of Novel Antioxidant L-Ergothioneine on the Sperm Functional and Mitochondrial Integrity In Normozoospermic Samples Post Thaw

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ABSTRACT

The objective of the present study was to assess the conservative effects of pre-freeze supplementation of L-Ergothioneine (EGT) in the cryomedia, on the post thaw mitochondrial membrane potential (MMP) and its associated functional parameters such as the motility, ability to undergo capacitation and acrosome reaction of human spermatozoa. Although sperm cryopreservation is an established technique, it is known to result in reduction of the above listed factors which would negatively affect the embryo quality and probability of blastocyst formation. EGT being an antioxidant, elucidates action against the ROS produced due to cryopreservation. In the present study, 400 normozoospermic semen samples were cryopreserved with (Test) and without (Control) EGT supplementation. Clinically relevant: Semen analysis (WHO 2010), *invitro* fertilization ability assessment (Chlortetracyclin Immunofluorescence study), MMP assessment (JC-1 immunofluorescence study) 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 quality and its mitochondrial integrity (Fresh vs Control: $p<0.001$). On addition of EGT, to the split of the same sample, significant conservation in the sperm quality was observed (Test vs Control: $p<0.001$). Further it was observed that with increase in ROS (assessed in MDA test), there was a regression in the MMP, motility and progression in the number of sperm which underwent premature capacitation and acrosome reaction which was significantly lower in the sperm split which was frozen post addition of EGT to the media. Hence the study provides proof that EGT acts against the ROS and conserves the spermatozoa's functionality.

Keyword: Sperm, Cryopreservation, EGT, Antioxidant, Sperm mitochondria

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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). With advancements in the science of Assisted reproductive technology (ART), the use of artificial aid which includes intrauterine insemination (IUI), *in-vitro* fertilisation (IVF) and intra cytoplasmic sperm injection (ICSI), are used primarily for treatment of infertility. The selection of the spermatozoa for use in ART was based solely on the subjective selection of morphologically normal and motile nature of it.

With the increase in awareness and treatment availability, there is an ever 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). Although sperm cryopreservation is an established technique, due to the thermal and osmotic shock the cell is exposed to, it causes reduction of mitochondrial quality.

The ATP generated by oxidative phosphorylation in the inner mitochondrial membrane is transferred to the microtubules, to drive motility (Zamboni *et al*., 1987) Therefore; an impairment of mitochondrial activity may explain the reduction in motility (O'Connell *et al*., 2002). Also, sperm mitochondria are known to uptake calcium, and have been hinted as a possible intracellular calcium store in human sperm (Costello *et al*., 2009), thus alteration in MMP activity can thereby alter the sperm's ability to undergo acrosome and capacitation which is the preliminary requirement for the sperm to fertilise the oocyte. The other associated cellular damage due to cryopreservation are studied in morphology (Nallella *et al*., 2004), plasma membrane structure and function (Andrea *et al*., 2010), apoptosis in frozen thawed spermatozoa (Paasch *et al*., 2004); and hence negative effect on embryo quality and probability of blastocyst formation (Braga *et al*., 2015)

The mechanisms behind the cryo-damage 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). Accordingly, a variety of cryoprotective media, most supplemented with antioxidants, have been employed in an attempt to overcome cryodamage (Yoshimoto *et al*, 2008; Li *et al*., 2010). Antioxidant supplementation has been shown to yield significantly improved quality of cryopreserved spermatozoa in experiments (Grossfeld *et al*, 2008). Hence, in this study, the structural and functional damage evaluation in sperm caused due to cryopreservation was analysed and the simultaneous assessment to study if the antioxidant supplementation (L-Ergothioneine) to the cryo media conserved the post thaw spermatozoa quality was done.

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, 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 mitochondrial integrity and thereby preserving the sperm motility and fertilisation ability. Following which effect of ROS level (as determined by MDA in sample) was correlated to the levels of alternation in sperm's functional paramaters. 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 Specialities 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.

Sample allocation

As seen is Figure I, the sample is split into three equal fractions. The first fraction (Fresh; Internal Control) was analysed for pre-freeze sperm qualitative and quantitative parameters. The remaining two parts are frozen in cryoprotectant (Glycerol Egg Yolk Media; GEYC) with (Test) and without (Control) supplementation of L-Ergothioneine (EGT). The frozen sample is then analysed for the same parameters to measure the effect of pre-freeze supplementation of novel antioxidant EGT on the post thaw survival and quality preservation in sperm.

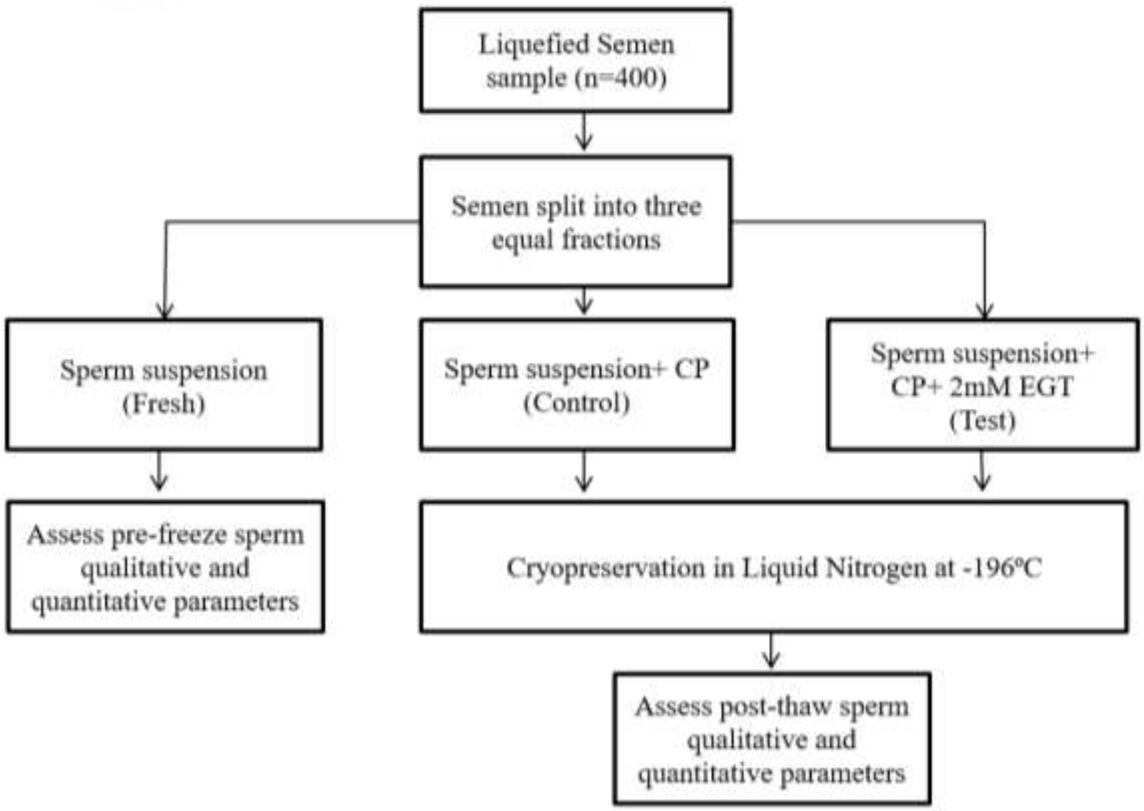

Figure I: Analysis of effect of L-Ergothioneine supplement, pre-freeze, on the post thaw functional integrity of human sperm.

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

Qualitative Analysis: Mitochondrial Membrane Potential, Lipid Peroxidation Status, ability of the sperm to undergo Capacitation and Acrosome reaction.

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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, as mentioned above, of the semen were cryopreserved in media with and without EGT by gentle mixing in the ratio 1:1. 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 optimization 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 split sample supplemented with 2mM EGT had significantly higher percentage of functional parameters and MMP post thaw when compared to control ($p<0.05$). Therefore, 2mM of EGT was used for further studies.

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

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.

Unlike the method mentioned in the WHO 2010 manual, 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 other tests were performed as mentioned in the WHO2010 manual wherein the number of motile sperm was 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 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 determined by assessing the occurring levels of thiobarbituric acid reaction (Rao *et al*., 1989; Esterbauer *et al*.,1990). The semen fraction was removed and the sperm was resuspended in phosphate buffer saline. Equal volumes of diluted semen were 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.

Capacitation and acrosome reaction status

Number of spermatozoa which underwent capacitation and acrosome reaction was assessed by CTC assay as described by Colas *et al*., 2009 and Perez *et al*., 1994 with modifications.

Capacitation and acrosome status were evaluated using slightly modified protocol of chlortetracycline (CTC) staining methods mentioned by Perez *et al*., 1994. Semen was centrifuged at 1000rpm for 5 min and 3µl of semen were mixed with 20 µl CTC working solution (2.42 gm Tris, 7.58 gm NaCl, 0.0604 gm cysteine and 0.0386 gm chlortetracycline at 100 ml distilled water. After 15 seconds, the reaction was stopped by addition of 10µl glutaraldehyde solution (12.5%) and slides were assessed under a fluorescence microscope (ECLIPSE, Nikon, Japan). Two hundred spermatozoa were evaluated and assigned to one of the following categories:

Un-capacitated: When fluorescence was detected over the whole region of the sperm head

Capacitated: When fluorescence was detected in the sperm head except in the post acrosomal region

Acrosome reacted: With no head fluorescence except for a bright band in the equatorial segment.

Determination of functional integrity of sperm mitochondria

Mitochondrial function is commonly monitored using cationic florescent probes which accumulate in mitochondria depending on the transmembrane electrical gradient. The MMP was assessed using JC-1 fluorescent dyes using the protocol adapted and modified from a study performed by Marchetti *et al*., 2004. Stock solutions of JC-1 (2mM) was made in Dimethylsulphoxide (DMSO) and stored in -20ºC. The subsequent working solutions were made to dilution 1:200 (1µM) and mixed with sperm suspension at 5 million/ml concentration. The entire mix was incubated at 37ºC for 30 minutes and the slides were viewed at 450-490nm on a fluorescent microscope (ECLIPSE, Nikon, Japan). A total of 200 spermatozoa were evaluated and assigns to one of the following categories.

Green florescence: JC-1 in monomeric form; depolymerised mitochondria; Low MMP

Red/Orange florescence: JC-1 in aggregate form; polymerised mitochondria; High MMP

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 this is a preliminary study, the effects of the novel chemical were initially assessed in semen that had normal values. There are various studies which mention the differential effect of cryopreservation on the abnormal to normal semen samples. To nullify the difference and to completely devote the results to the effect of EGT, specifically only normozoospermic (according to WHO 2010) samples were considered in this research.

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.

Cryopreservation induces regression in sperm quality post thaw

The freeze thaw procedure protocol, even though is standardised, was observed to cause reduction in the overall quality of the sperm, thus making it suboptimal to use. The important parameters such as motility, ability of the sperm to fertilise and the mitochondrial potential were compromised when compared to the fresh split assessed (Fresh vs Control: $p<0.05$). The peroxidation of the sperm lipids was higher post thaw pointing to the loss of structural and functional integrity of the cell (Table III).

Table III: Parameters analysed with and without supplementation of EGT

Parameter (N=400)	Fresh (Internal Control)	GEYC (Control)	GEYC+2mM EGT (Test)
Total Motility (%)	56.6±1.4	36.5±9.1	48.3±12.7a
Progressive Motility (%)	38.5±6.3	21.6±5.6	30.7±5.6a
Viability (%)	70.4±1.4	35.1±3.5	59.8±7.1a
Capacitation (%)	56±11.2	66.6±4.2	58.07±12.7a
Acrosome reaction (%)	44±10.3	60.8±11.3	47.6±12a
MMP (%)	64.5±9.36	45.5±3.5	57.1±5.6a
MDA (ng/ml)	0.9±0.09	6.9±2.3	2.7±1.7a

(a) $p<0.05$: Significant difference observed among all parameters: Test vs Control

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

Different concentrations of EGT, when supplemented to the cryo-media prior to freezing, had varied effects on the sperm quality post thaw (Table I). Interestingly it was observed that the with ascending concentrations, above 2mM (2.5mM, 3mM and 3.5mM), there was a rapid decline in the post thaw parameters. Hence the concentration of EGT was fixed to not more than 2Mm for use in human sperm cryopreservation. Further, more clinically essential tests were performed at a specified concentration of 2mM to confirm the use of the antioxidant in day-to-day basis in cryopreservation.

observed among all parameters: 2mM vs Control

Effect on sperm 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 EGT when compared to the part which was frozen without it. The test group showed higher preservation ($p<0.05$) of viability (Control vs 2mM: 35.1±3.5 vs 59.8±7.1) when compared to control group.

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 was lesser oxidation of sperm ($p<0.05$) when compared to control (Control vs 2mM: 6.9±2.3 vs 2.7±1.7); indicating that the oxidation of the lipids is higher in the absence of the antioxidant.

Effects on MMP thereby on the dependent motility:

The levels of activity potential were analysed using JC-1 staining (Figure II), here the number of spermatozoa showing orange fluorescence were counted and hence the percentage of sperm having intact mitochondria in the absence of supplementation were observed to be 45.5±3.5 which was lower than the ones which contained the addition if EGT pre freeze, which showed a MMP of 57.1±5.6.

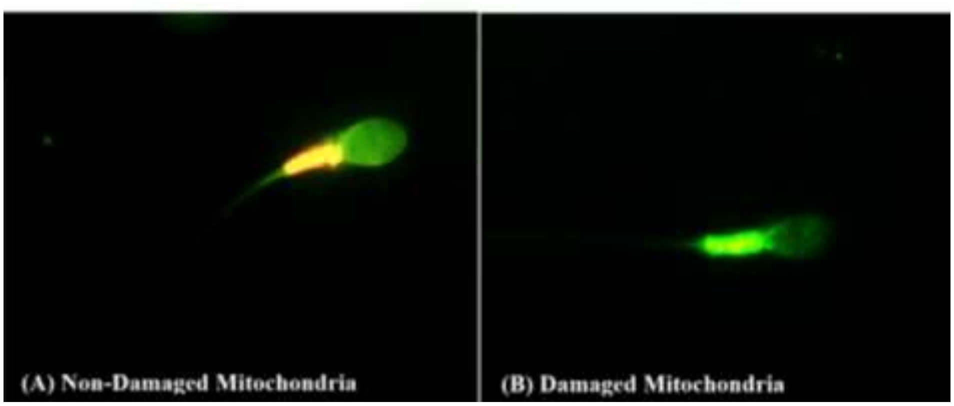

Figure II: Mitochondrial Membrane Potential (%) - JC1

On addition of JC-1, the intact mitochondria (polymerised) forms aggregates with the dye to give red fluorescence at 590nm. In (B), the JC-1 reagent disperses through the entire sperm due to the depolymerised structures of mitochondria, thus giving a green fluorescence at 527nm.

Also the motility which was depended on the MMP, seemed to show a similar trend of drop in the number of sperm which retained the quality of motility when compared to the control split of the same sample. The total motility (Control vs 2mM: 36.5±9.1 vs 48.3±12.7) and progressive motility (Control vs 2mM: 21.6±5.6 vs 30.7±5.6); thus had significant loss of kinetics ($p<0.05$).

Effect on the fertilisation capability

The ability of the sperm to fertilised is assessed *invitro* by specific tests which involved the study of the ability of the sperm to undergo capacitation and acrosome reaction pre and post thaw. As seen in Figure III, the reaction is measured by the ability of the sperm to selectively stain the posterior and anterior head region. It was observed that the number of sperm which underwent the premature reaction were lower in sperm frozen in the presence of antioxidant when compared to freezing in the absence of it; i.e. Capacitated sperm (Control vs Test: 66.6±4.2 vs 58.1±12.7) and Acrosome reacted sperm (Control vs Test: 60.8±11.3 vs 47.6±12) were of statistically significant ($p<0.05$) levels.

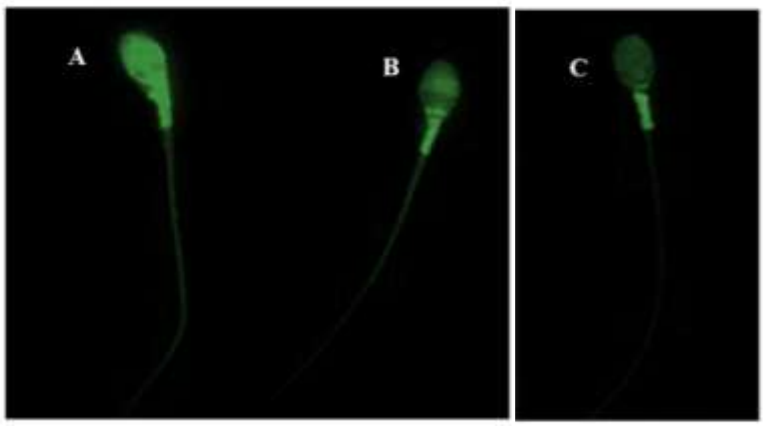

Figure III: *Invitro* fertilization assessment in spermatozoa using Chortetracycline fluorescence assay

(A) Spermatozoa displaying uniform fluorescence on the head which is characteristic of uncapacitated spermatozoa. (B) Sperm showing prominent fluorescent positive equatorial segment, mid-piece of the tail and fluorescence-free (dark) band in the post-acrosomal region is typical of capacitated spermatozoa. (C) Sperm showing low fluorescent signal throughout the sperm head, with remaining positive signal in the equatorial segment and mid-piece is typical of acrosome reacted spermatozoa.

Correlation between ROS levels (MDA ng/ml) with Mitochondrial Membrane Potential (%)

It was observed that the increasing levels of ROS generated in the semen during cryopreservation had a significant correlation ($p<0.05$) with the decrease of Mitochondrial Membrane Potential (Negative Correlation).

Figure IV shows a positive correlation with r= -0.119 (p=0.018*) on correlating the ROS generated with the MMP in sperm in the Test group, thus confirming the correlation between the levels of ROS and the mitochondrial potential of the sperm.

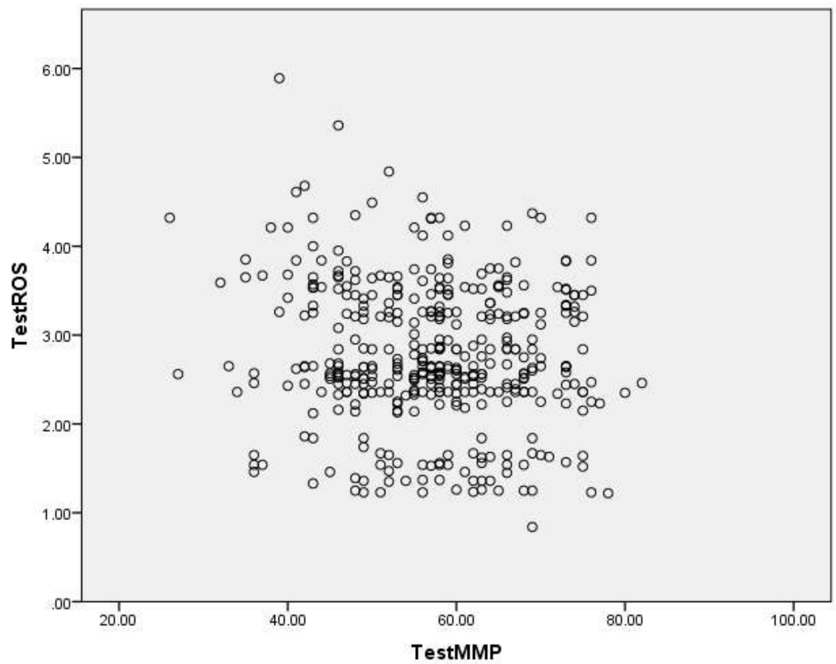

Figure IV: Correlation of ROS levels (MDA ng/ml) with MMP (%)

The above figure shows a negative correlation with r= -0.119 (p=0.018*) on correlating the ROS generated with the Membrane potential of the mitochondria in the Test group.

Technique of human semen cryopreservation is an important technique routinely employed in the clinical management of male infertility (Medeiros *et al*., 2002). Sperm possess cellular organelles; the susceptibility of which to freeze–thawing damage may differ depending on accessibility to cryoprotectant (O'Connell *et al*., 2002). Sperm possess both plasma and mitochondrial membranes and susceptibility to freeze–thawing damage may differ depending on accessibility to cryoprotectants. Thus the level of damage in the

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.

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).Even though the above mentioned negative correlation between ROS and IVF fertilization rate has been found (Zorn *et al*., 2003), 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). 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).

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), superoxide anions and singlet oxygen production (Obayashi *et al*., 2005) at that particular measure.

Çoyan *et al* (2011) 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.

It is becoming increasingly apparent that mitochondria are initiators of cell death by apoptosis (Dinsdale *et al*., 1998; Green and Reed, 1998; Sun *et al*., 1999). In correlation with this, the study suggests that the parameters measured here are interdependent and that functional plasma and mitochondrial membranes are necessary to maintain motility an d ability of the sperm to undergo capacitation and acrosome reaction; the quality of which seemed to be much conserved on addition of EGT pre-freeze.

Similarly, various studies have deliberated in detail and established the fact that the fertilisation capacity of the human spermatozoa was found to be preserved on addition of antioxidant (Chi *et al*., 2008). The important functional aspect of the spermatozoa is the occurrence of certain chemical reactions which are necessary for the sperm to fertilise at the vicinity of the oocyte. On addition of EGT, it was seen that there was a reduction in the amount of spermatozoa with functional damage. 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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