IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

EVALUATION OF BIOTIN AND PENTOXIFYLLINE COMBINATION ON SPERM MOTILITY AND QUALITY IN ASSISTED REPRODUCTIVE TECHNOLOGY

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Abstract:

Objective: The study aims to carry out the motility of spermatozoa plays a crucial role in determining the appropriate infertility treatment strategy and directly impacts the success rate in assisted reproductive technology (ART). Semen samples from participants were taken and divided into three groups biotin treated and pentoxyfilline treated.

Methods: Semen samples from the motherhood fertility center (n=65) were cryopreserved in liquid nitrogen using a cryoprotective solution buffered with Human serum albumin. The semen samples were post-thawed after 2 weeks and the motile spermatozoa were extracted using the HSA medium supplemented with either pentoxifylline (1nM) or biotin (10nM). After incubation, the post-wash motility was observed for up to 4 hours.

Results: Supplementing with biotin and pentoxifylline increased motility overall ($p<0.01$), progressive ($p<0.01$), and motility overall for ($p<0.05$) progressive ($p<0.05$) v/s biotin and $p<0.05$ for pentoxifylline respectively. At 4 hours the percentage was found to be 35%. Higher concentrations of the substance (particularly 100nM) generally result in a higher percentage of spermatozoa over time. Total motility shows significant increases post-thaw ($P<0.001$). Progressive motility increases post-thaw except for a slight decrease in asthenozoospermia. Slightly decreases or remains unchanged post-thaw with a significant decrease in teratozoospermia. At all-time points, there was no discrete variation in the motility between the biotin and pentoxifylline groups (Figure 1).

Conclusion: Biotin and pentoxifylline combination might be a secure and useful substitute medium to improve the quality of frozen sperm in an assisted reproductive technology (ART) system.

Keywords: Pentoxifylline, asthenozoospermia, teratozoospermia, assisted reproductive technology, post-thaw

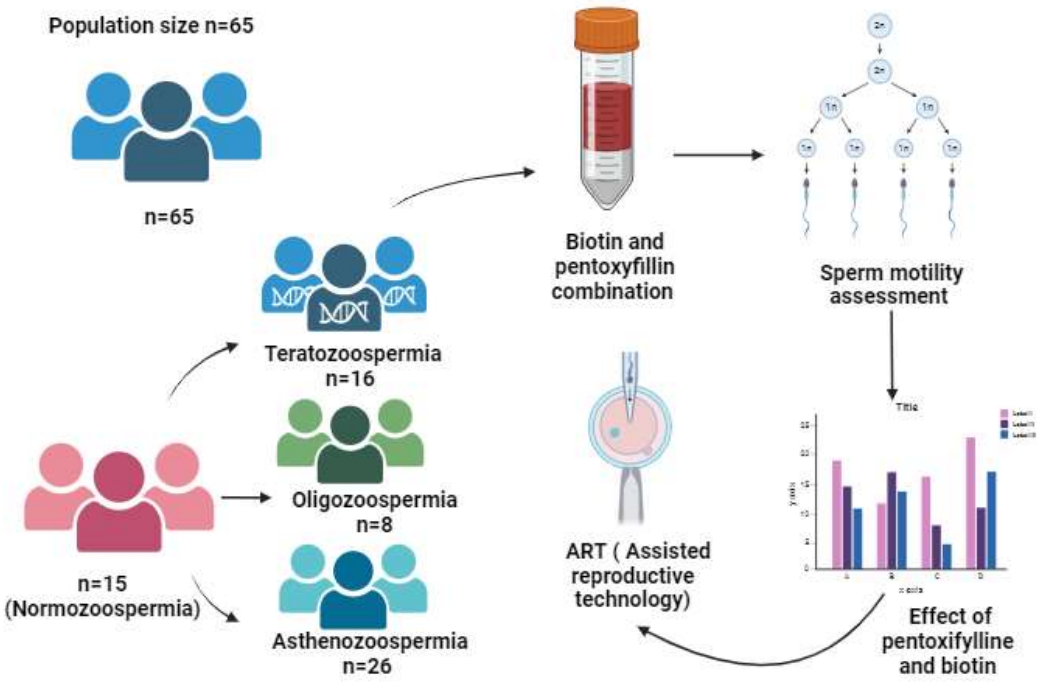

Figure 1: Schematic diagram representing normozoospermic, asthenozoospermic and assisted reproductive technology

I. INTRODUCTION

Over the global adult population of 17.5 %, approximately 1 in 6 couples worldwide suffer from infertility. Among them, 15-20 million (25%) alone are in India. According to a report by the World Health Organization (WHO), (Mascarenhas, 2012). Infertility is a precondition of the human reproductive system where couples are unable to conceive even after one to three years of waiting period. (WHO, Katole, 2019). Sperm motility is one of the most important functions involved in assessing infertility in male, concerning their age group and lifestyle adaptability toward their daily dietary intake. The whole procedure behind preserving the semen sample involves many cytoplasmatic and exocytosis changes upon exposure to cryoprotectants, freezing, and thawing (Kalthur, 2008) (Figure 1). Sperm motility is referred to as the movement ability of individual sperm to swim effectively so it can reach the egg called “Normospermia”. The other condition where the ability of sperm is low toward motility is asthenospermia any damage to the testicles or defect in sperm flagellum can lead to such a condition (Leslie, 2024). Sperm count in fertility also relates to healthy conception, below 15 million sperm per milliliter of the semen sample leads to oligozoospermia (Hussein , 2018) A healthy-looking sperm has an oval shape with a normal size appearance, any deviation in the shape and morphology of sperm is referred to as teratozoospermia (Atmoko , 2022 & Leslie, 2024). Healthy living is one of the major factors for natural pregnancy in couples without any medical assistance (Emokpae, 2021). Cryopreservation of spermatozoa over the recent advanced technology for fresh and frozen semen samples has shown progressive motility and enhancement for the fertility condition in couples (Tamburrino, 2023).

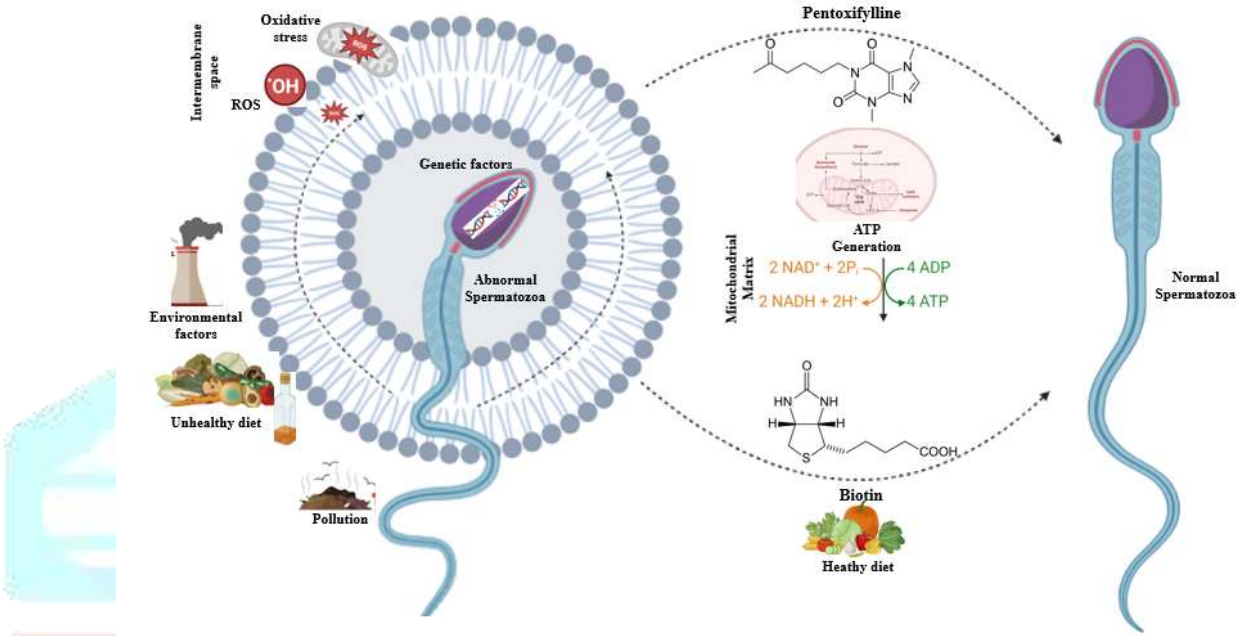

Figure 2: Schematic representation involving Cryopreservation of spermatozoa and progressive motility

Cryopreservation of human spermatozoa has progressed and is an important part of assisted reproductive technology (ART). Many factors affect the motility of sperm and quality of sperm concerning assisted reproductive technology using cryopreservation techniques. The fertility treatment related to ART involves a procedure where the eggs are surgically removed and fused with male gamete under paramount laboratory conditions and later injected back into the woman's womb for further resumption of pregnancy (Henkel, 2003). ART is one such technique where several techniques are involved for treatment-related infertility in couples. Pregnancy can be owned in different methods assisting ART such as Invitro fertilization (IVF), Intracytoplasmic sperm injection (ICSI), intrafallopian transfer, and intrauterine insemination (IUI) (Figure 2).

Several studies have revealed that seminal oxidative stress (OS) and reactive oxygen species (ROS) play a major role in sperm motility and production concerning male fertility (Takeshima, 2021). Oxygen has a vital role in biological systems either positively or negatively, the major function is related to oxidative phosphorylation where in synthesis of adenosine-5-triphosphate (ATP) molecules takes place from mitochondria (Manjula, 2013 & Burton, 2011). Moderate level of ROS and reactive nitrogen species (RNS) at the cellular level plays an important role in the signaling pathway and metabolism. Increased levels of concentration in ROS/RNS can lead to oxidative damage of nucleic acid and fragmentation in DNA (Kruk, 2019). Apart from the functional role of ROS and RNS generation in the biological system, extreme ROS regulation, and oxidative stress, nitrogen species can lead to harmful effects on human spermatozoa resulting in morphological and cellular disruption causing infertility (Agarwal et al., 2008). Recent advancements in research have revealed that altered redox balance between the seminal fluid and reactive oxygen species can affect sperm stability leading to infertility. An increase in blood vessels and plasma level redox potential leads to infertility (Majzoub , 2018).

All the living creatures in the environment find natural sources for defense mechanisms as antioxidants to scavenge all the free radicals present concerning reactive oxygen species and oxidative stress-causing

molecules. Antioxidants help in the neutralization of free radicals and the presence of antioxidants in the seminal plasma leads to protection from any detrimental causes of ROS generation (Qamar, 2023). The Biotin Vitamin (B7) complex plays a crucial role in sperm motility and longevity in the frozen-thawed cryopreserved as well as fresh spermatozoa (Saliana, 2019). Pentoxifylline acts as a bronchodilator, a derivative of xanthine that helps in sperm viability and motility (Ghasemzadeh , 2016). Most familiar non-enzymic antioxidants such as ascorbic acid, biotin Vitamin B7, pentoxifylline Vitamin E, Vitamin C, glutathione, and tocopherols (α, β, γ, and δ) Vitamin E acts as an important molecule to fight against free radicals. Biotin and pentoxifylline acts as a potent antioxidant organic compound to scavenge against free radical and oxidative stress, concerning its wide-range property that helps in the disruption of free radicals present in the semen sample and also helps in preventing the overproduction of lipid peroxides and superoxide molecules to protect human spermatozoa from any external oxidative damage (Young, 2001 & Devi, 2004). Moreover, Biotin and pentoxifylline augmentation is shown to enhance DNA integrity and low-motile sperm after post-thaw. Supplementation of antioxidant molecules to both the samples fresh and cryopreserved media formerly progresses the post-thaw survival and DNA damage of spermatozoa. Generally, Vitamins have a very crucial role in protecting the cellular membrane from any external damage impacting male fertility (Zempleni, 2012 & Nabi, 2017). This Vitamin also helps in improving sperm motility and Boosting male fertility (Taylor, 2009).

Cryopreservation of semen samples after vitamin E and vitamin B7 supplementation significantly improves sperm motility in post-thaw and fresh-ejaculated spermatozoa (Ozimic, 2003). The major possibility of using biotin vitamin B7 and pentoxifylline vitamin E supplements as an antioxidant may improve the frozen and fresh spermatozoa semen sample (Kalthur, 2012). The major aim of our study was to determine the effect of biotin and pentoxifylline addition to cryoprotective media on post-thaw motility and DNA damage in patients with asthenozoospermia condition where motility of spermatozoa is low in men with anomalous spermatozoa sample.

II. MATERIALS AND METHODS

2.1 Sample collection

The study was conducted on patients undergoing fertility treatment (n=65) at Motherhood Hospital from October 2022 to December 2023. Those patients were randomized into two groups (mean age 32.28 ± 2.5 years) their semen samples were analyzed. Written consent for the research was given by the subjects. The sample collection was done to check four different conditions. In normozoospermic(n=15), oligozoospermia (n=8), asthenozoospermia(n=26), and teratozoospermia(n=16) cases, samples were collected by men who had abstained using Makler's counting chamber and the sperm density was determined following the liquefaction of the ejaculate. The morphology and motility of the sperm were evaluated according to WHO standards. The ethical instituitional committee was taken from Institutional review board for the use of human material in women's and Children hospital. (WHO protocol).

2.2 Optimization conditions for sperm samples supplementation

The ejaculated sperm samples collected from oligoasthenospermia, asthenozoospermia, and teratozoospermia males were assessed after supplementation with biotin and pentoxifylline where HEPES buffer was added, and later in HSA media (125ml) at +2ºC to 8ºC sterilized using aseptic condition. The samples were separated into equal-volume aliquots for washing it with top of the pellets performed using a Kitazato kit (Kovacic, 2006).

2.3 Semen sample preparation induced using biotin as a supplement

The frozen materials were separated into three equal portions and quickly thawed at 37ºC after 2 weeks of conditions. They combined the sperm suspension with 2 ml of HEPES supplemented with HSA media from Vitrolife and centrifuged at 1800rpm to remove the motile spermatozoa from the pellet. About 200-300 µl of HEPES was mixed with HSA and 10nM biotin was gently placed over the sperm pellet (Kalthur, 2008).

2.4. Preparation of semen sample induced using pentoxifylline as a supplement

A stock solution (1mg/ml) of pentoxifylline (Kitazato, India, Cat. No.94215, PF-05×5, 0.5ml×5 was prepared using Calcium chloride, gentamycin glucose and HEPES. Semen ejaculates of normozoospermic

men were supplemented with different concentrations of pentoxyfilline(0.5ml×5), (1nM, 5nM,10nM, 50Nm,100nM) and then mixed with equal amounts of sperm to the pentoxyfilline. After the sperm sample was added 10mL of the sepasperm wash solution was added. After centrifuging for 5 minutes at 200×g pick up the sperm pellet and move on to the IVF procedure.

2.5. Sperm preparation for motility

The motility of the sperm was determined after the incubation preparation medium at 37 °C. The impact of biotin supplementation was evaluated for 1, 2, and 4 hours after incubation at 5% CO2. A clean glass slide was carefully covered with a coverslip, and approximately 10 µl of spermatozoa were divided into 4 groups to check motility rapidly progressive (grade a) slow or sluggish (grade b), non-progressive (grade c), and immotile (grade d) spermatozoa were observed using light microscopy (100x) magnification. (Mahadevan, 2008).

2.6. Classification of split ejaculate

Sperm samples were collected from asthenozoospermic patients and divided into groups. Two containers were labeled as fraction 1 and fraction 2 were collected as semen samples. Each participant was directed to fill the jar as
fraction1, with material collected in the second container. The same patients were given 6 days to collect samples from patients regarding sample collection. In liquefied semen, sperm count, motility, and morphology were evaluated using World Health Organisation standards (WHO, 1992, 1999). Sperm density was measured using >20 million mL of cells, sperm motility was observed to be greater than 40%, and normal morphology was found to be greater than 30% which was considered typical for semen samples. Samples of semen containing 1×10^6 WBC cells/ml. The impact of reactive oxygen species (ROS) on sperm motility was investigated. (Kumar, 2011).

2.7. Sperm DNA integrity

The study evaluated for mitochondrial membrane potential and DNA integrity of spermatozoa for 24 and 48 hours post-incubation. A minimum of 400 spermatozoa were collected and scored per sample, and results were expressed as percentages. DNA integrity was evaluated using the sperm chromatin dispersion (SCD) test, which categorized sperm heads into three groups based on halo size observed under a fluorescent microscope no halo, small halo, and large halo. Spermatozoa with no halo or small halo were considered to have DNA damage and the results were expressed as percentages. (Aditi, 2012).

2.8. Eosin and Nigrosin staining

The ability of sperm to undergo capacitation was assessed in frozen-thawed using spermatozoa by eosin and nigrosine staining as described by (Ashok Agarwal and Sajal Agarwal) with minor modifications. Place a drop of well-mixed semen on a boerner slide. Add 2 drops of 1% aqueous eosin. After that add 2 drops of 10%aquoues nigrosine. Mix well with a wooden stirrer in the Boener slide well. The coverslip was placed using mounting media. The nigrosine provides a dark background that makes it which is easier to observe faintly pink heads and dead spermatozoa have heads that are stained red or dark pink. Viability in normal sperm were specimens at 95% CI. At least 200 spermatozoa were scored from each sample and classified as non-capitated and dead spermatozoa. (Ashok, 2009).

2.9. Biochemical Assays

2.9.1. Lipid Peroxidation Measurement

To test the amount of oxidative stress causing damage to spermatozoa samples collected from the patients with normozoospermic and asthenozoospermic by using the TBARS method several other techniques also imply for testing the level of lipid peroxidation like Malondialdehyde, 4-hydroxynoenal Histidine, Isoprostane.

2.9.2. Thiobarbituric acid-reactive substance (TBARS)

Oxidative stress in the cells is the indication for lipid peroxidation. Thiobarbituric acid is a reactive substance assay that helps measure aldehyde lipid hydroperoxides and malondialdehyde. A combination of MDA and TBA is formed to formulate a fluorescent compound. Sample preparation was done for spermatozoa adjusting the concentration of cells of 10^6/ml. Concisely 1 ml of the sample was added with TBA containing 3ml reagent containing trichloroacetic acid and HCL. The whole chemical composition was mixed well and treated in a water bath at a temperature held for 10-15min duration. Later after cooling the mixture, it was subjected to centrifugation at 6000rpm for 10min. Retaining the pellet, the absorbance of the samples was taken at 530-570nm(Katole , 2009).

2.9.3. CAT activity

Catalase activity was determined by the method[20]. Catalase is a universal antioxidant enzyme that helps in the degradation of hydrogen peroxide into oxygen and water molecules concerning oxidative stress which is measured by performing spectrometric analysis and recording the absorbance at 240nm indicating a decrease in the enzyme activity representing catalase activity. A mixture of hydrogen peroxide is diluted using phosphate buffer by maintaining pH 7 with its initial optical density between 0.5 and 0.6 units of absorbance at 240nm at the time duration of 30 sec maintaining temperature at 25°C. CAT activity was noted and calculated from the change in absorbance and expressed as µg/mL.

2.9.4. Tocopherol- Total antioxidant capacity (TAC)

Tocopherol-containing antioxidant properties have been reportedly used to test the potential of enhancing prognosis for infertile couples. Supplementation of tocopherol concerning temperature has been tested to improve the motility and viability of spermatozoa carrying out TBARS and CAT activity enzymatic study concerning oxidizing agents present due to oxidative stress. Results signify ROS-MDA-CAT-TAC capacity to infer the level of supplementation of antioxidants such as tocopherol produced as an effective of all seminal constituents concerning proteins, vitamins, and lipids components (Figure. 3)(Kumar ,2019).

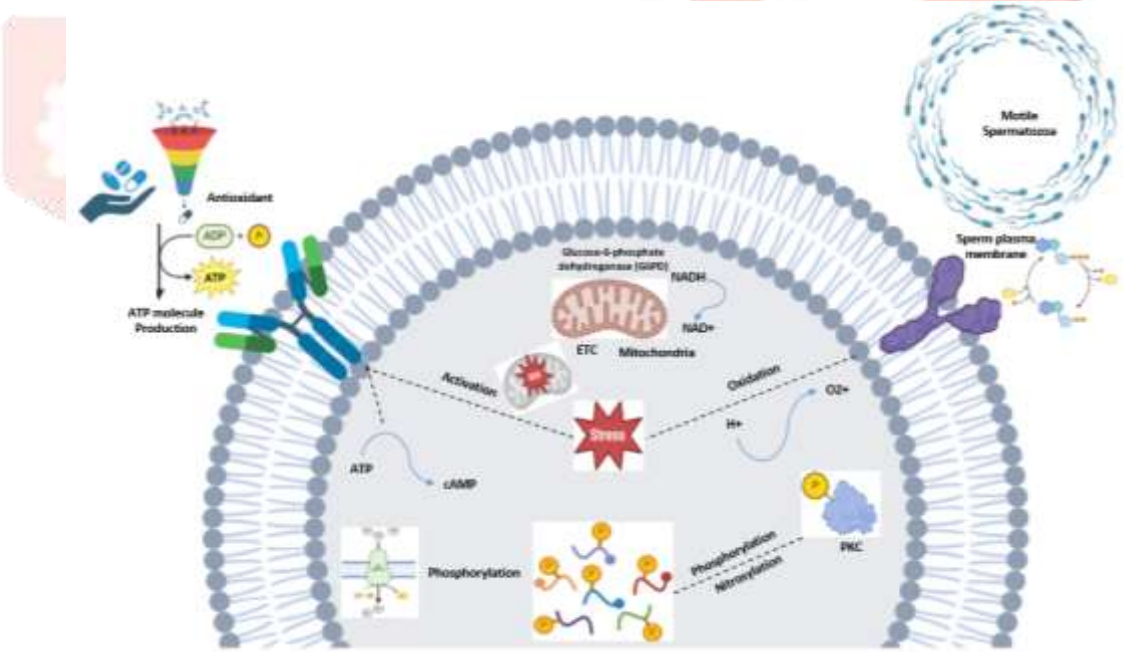

Figure 3. Illustration of the damage caused by reactive oxygen species and nitrogen molecules towards the production of oxidative stress triggering the functional parameters of spermatozoa and antioxidant supplementation towards cryopreservation of spermatozoa sample.

2.9.5. Assessment of seminal plasma Superoxide dismutase (SOD) activity

Oxidative stress causes damage to the spermatozoa cells, SOD plays a key role in antioxidant enzyme detection which protects the sperm seminal plasma level from oxidative stress. The nitrite inhibition reduction method is due to the superoxide anion, which is generated after the conjunction of xanthine and xanthine oxidase. The activity of SOD was measured by the amount that caused 50% inhibition in the rate of nitrite reduction expressed in the U/ml unit seminal plasma level (Kumar, 2009).

4. Statistical analysis

The motility data were analyzed using one-way ANOVA and displayed as mean±SE using the Graphpad Instat 9.0 software. Using the Bonferroni technique multiple comparisons were conducted, and the values with a significance level of $P<0.05$ were deemed statistically.

III. Results:

Among the 65 participants samples 15 were normozoospermic, 8 were oligozoospermic, 26 were asthenozoospermic and 16 were teratozoospermic. Figure 1 represents the percentage of spermatozoa at different concentrations of 1, 5, 10, 50, and 100 nM of sample concentration where treatment was measured at various time intervals during 1hr, 2hr, 3 hr, and 4 hrs after incubation.

3.1 Optimization conditions for sperm samples supplementation

3.2.1. Semen sample preparation induced using biotin as a supplement

The study found that a sperm concentration of 50nM with 60% motility had the highest proportion of spermatozoa, which tended to decline over time. Specifically, 60% motility group has the largest proportion of spermatozoa, followed by the 100nM or 55% motility groups. The percentages of motile spermatozoa, followed by the 100nM or 55% motility groups. The percentages of motile spermatozoa were lower after 4 hours compared to the initial measurements but the 50nM concentration continues to display the greatest percentage of 42% compared to the other concentrations. Different letters indicate statistically significant differences between the groups. At each time interval, significant difference was observed with respect to concentration at each point of time interval. It was found that the concentration of the treatments (biotin and pentoxyfilline) affected the viability of spermatozoa over time, with higher concentrations generally maintaining higher percentages of viable spermatozoa, especially in the first few hours of incubations. (Figure 4).

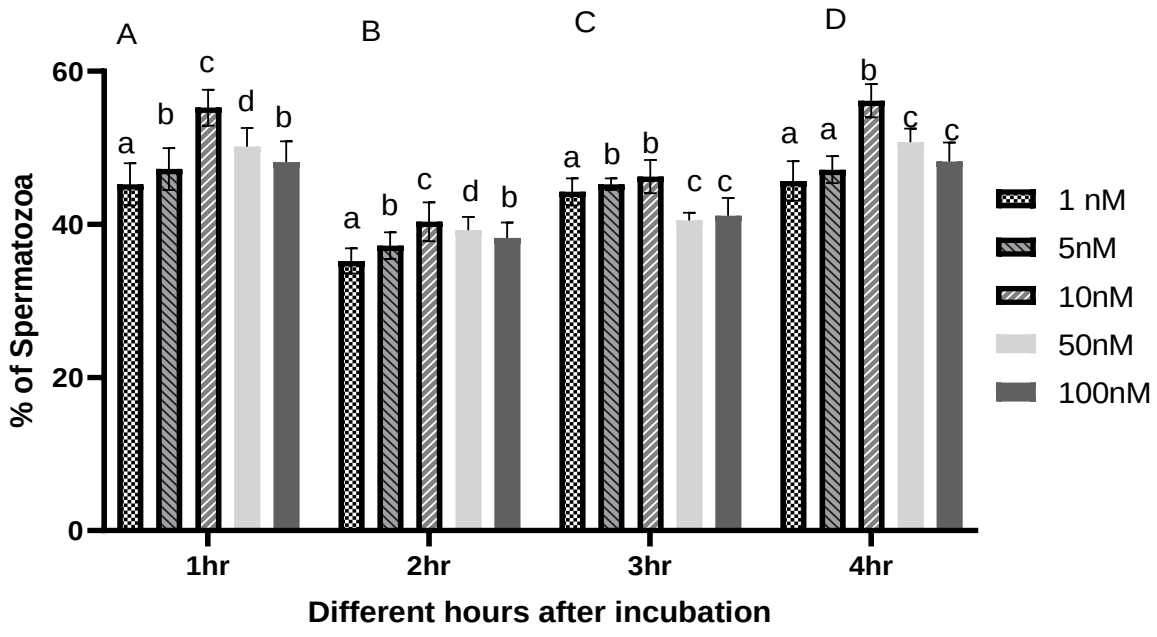

Figure 4. Percentage of spermatozoa and the effect of biotin was compared in 1 - 4 hrs. Normozoospermia (n=15), asthenozoospermia (n=26), teratozoospermia (n=16) and oligozoospermia (n=8). Values are means

±SE (n=65). Different concentrations on per hour 1nM, 5nM, 10nM, 50nM and 100nM. Motility of sperm was determined using one-way ANOVA between trials and groups followed by the Bonferroni multiple comparison test and considered significant at $p<0.05$. Changes were significantly different between each group

3.2.2. Preparation of semen sample induced using pentoxifylline as a supplement

The percentage of spermatozoa at different concentrations of a substance (1nM, 5nM, 10nM and 100nM) over various incubation times (1, 2, 3, and 4 hrs). The graph also uses different letters a, b, c indicate statistically significant differences between groups within each time point. At 1 nM and 5 nM concentration, the percentage of spermatozoa was around 45% and those two groups are not significantly different from each other both labeled a. Bar graph 2 (Figure 2) shows at 10nM, the percentage increases to about 50% considerably higher than 1nM and 5nM (labeled b) at 50nM and 100nM the percentages are around 55% and 60% respectively significantly higher than 10nM when labeled as c. After 2 hrs the percentage dropped to about 35% labelled as 5nM the percentage is slightly higher around 38% labeled as a, whereas at 10nM the percentage is about 40% labeled as b significantly different from 1nM and 5nM. After 3 hours, the percentages are around 40% and these two groups are not significantly different from each other both labelled as a. At 10nM the percentage increases to about 45% which is significantly higher than 1nM and 5nM (labelled a and b). At 50nM and 100nM the percentages are around 48% and 50% respectively with 50nM significantly different from the lower concentrations (labeled b) and 100nM significantly higher than 10nM (labelled c). At 4 hours the percentage is about 35% (labelled 'a'). At 1nM the percentage is about 35% labeled a, at 5nM the percentage is slightly higher around 40%labeled a. At 10nM the percentage is around 42% significantly higher than 1nM (labeled b). Overall, the graph indicates that higher concentrations of the substance generally result in a higher percentage of spermatozoa over time, with significant differences marked by different letters at each point (Figure 5).

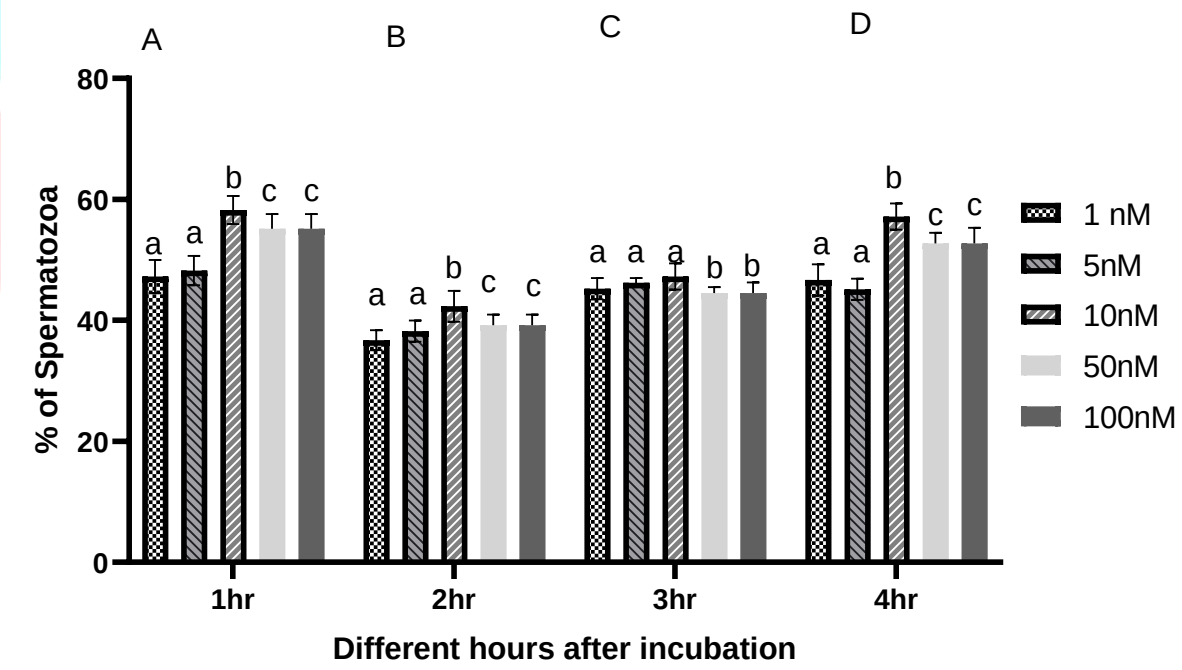

Figure 5. Percentage of spermatozoa and the effect of pentoxyfillin was compared in 1 - 4 hrs. Normozoospermia (n=15), asthenozoospermia (n=26), teratozoospermia (n=16) and oligozoospermia (n=8). Values are means ±SE (n=65). The motility of sperm was determined using one-way ANOVA between trials and groups followed by the Bonferroni multiple comparison test and considered significant at $p<0.05$. Changes were significantly different between each group

3.2.3. Sperm preparation for motility

The percentage at different concentration of a substance (1, 5, 10, 50 and 100nM) various incubation times at 1, 2, 3 and 4 hours. The graph also uses different letters (a, b and c) to indicate statistically significant differences between groups within each time point.(Figure 3) At 1nM, 5nM and 10nM the percentage of spermatozoa is around 45% and these three groups are not significantly different from each other all labelled as a. At 50nM and 100nM the percentages are around 55% and 60% respectively, significantly higher than the lower (labelled b). At 2 hours 1nM, 5nM, 10nM and 50nM the percentages of spermatozoa are around 35%to 40% and these groups are not significantly different from each other (all labelled 'a'). At 100nM the percentage increases to about 45% which is significantly higher than the lower concentrations (labelled b). At 1,5,10 and 50nM the percentages of spermatozoa are around 40% to 45% and these four groups are not significantly different from each other (all labeled a). At 100nM the percentage increases to about 50% which is significantly higher than the lower concentrations (labelled b) (Figure 6).

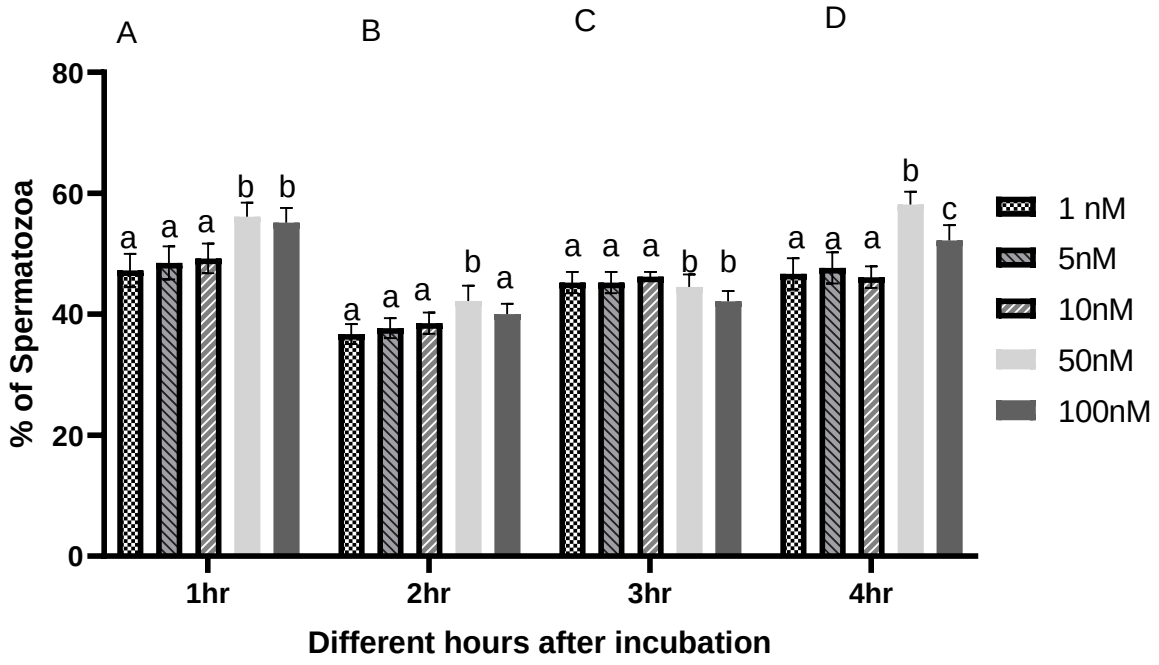

Figure 6. Percentage of spermatozoa and the effect of a biotin + pentoxyfillin was compared in 1 - 4 hrs. Normozoospermia (n=15), asthenozoospermia (n=26), teratozoospermia (n=16) and oligozoospermia (n=8). Values are means ±SE (n=65). The motility of sperm was determined using one-way ANOVA between trials and groups followed by the Bonferroni multiple comparison test and considered significant at $p<0.05$. Changes were significantly different between each group.

3.2.4. Classification of split ejaculate

At 1, 5, 10, and 50nM the percentages of spermatozoa are around 35% to 40% and these groups are not significantly different from each other. At 100nM the percentage increases to about 55% is significantly higher than the lower concentration (labeled c). Overall, the graph indicated that higher concentrations of the substance (particularly 100nM) generally result in a higher percentage of spermatozoa over time with significant differences marked by different letters at each point. (Table 1).

Table 1: Features of sperm incomplete motility and split fractions motility

Groups	Motility of spermatozoa (%)			Abnormality observed in the spermatozoa (%)			Viability (%)		
	FCE	SF1	SF2	FCE	SF1	SF2	FCE	SF1	SF2
Control	51.14±2.24	54.01±1.71	52.91±1.33	95±1.8	96±0.6	95.40±1.0	64.12±2.3	66.48±1.8	65.21±1.2

Motility of spermatozoa (%) for FCE (fresh control ejaculate), SF1 represents (spermatozoa fraction 1), SF2 represents (spermatozoa fraction 2) $^{*}P < 0.01$ represents statistically significant differences in the treatment group.

3.2.5. Sperm preparation motility

The Table 2 results outlines three key parameters related to spermatozoa across different groups motility, abnormality, and viability. These are compared among three groups control, FCE, SF1 and SF2. The motility of sperm observed slight variations across the FCE, SF1, and SF2 groups with SF1 showing the highest motility percentage at 54.01±1.71. However, SF1 consistently has the highest percentages in motility and viability while it also shows a slightly higher percentage of abnormality. Further analysis could help determine if these differences are statistically significant and what time they imply about the conditions or treatments represented FCE, SF1 and SF2.

Table 2: Features of sperm incomplete motility and split fractions motility

Groups	Progressive motility of spermatozoa (%)			Abnormality observed in the spermatozoa (%)			Viability (%)		
	FCE	SF1	SF2	FCE	SF1	SF2	FCE	SF1	SF2
Normozoospermia (n=15)	47.24±2.74	47.04±2.74	46.94±2.74	92±1.8	93±1.6	93.40±1.8	73.72±2.3	71.48±1.8	63.21±3.2
Oligozoospermia (n=8)	46.67±2.57	46.07±2.57	45.57±2.57	95±1.8	96±1.6	95.40±1.8	56.4±5.4	63.8±5.8	57.6±6.2
Asthenozoospermia (n=26)	36.72±1.65	35.72±1.65	35.02±1.65	95±1.8	95±0.8	94±00	53.4±2.3	52.4±2.3	43.21±6.4
Teratozoospermia (n=16)	45.25±1.76	44.25±1.76	46.25±1.76	97±1.8	98±1.0	97±5.8	56.54±2.3*	62.25±3.1*	51.0±5.7

*p < 0.01 represents statistically significant differences

Motility of spermatozoa was observed in post thaw semen samples cryopreserved after the addition of split ejaculate (100 µM) to semen samples where p<0.01 when compared to fresh control ejaculate of respective groups of SF1 and SF2 of semen samples of normozoospermic subjects. Abnormality was observed in SF2 was found to be slightly higher.

3.2.6. Sperm DNA integrity and Viability

Normozoospermic sperms for Progressive motility of the sample FCE, SF1, and SF2 are similar, abnormality for SF2 was found to be slightly higher. Abnormality SF2 had slightly higher. Viability for FCE is the highest followed by SF1 and then SF2 (Table 3). For Oligozoospermic patients, progressive motility was seen in FCE, SF1, and SF2 slightly higher. Viability for FCE is the highest followed by SF1 and then SF2. Oligozoospermic patients were found to be 8 for progressive motility for FCE, SF-1, and SF2 was slightly higher. Viability for FCE is the highest followed by SF1 and then SF2. For oligozoospermic sperms was found to be n=8, where progressive motility for FCE, SF1, and SF2 are similar. Abnormality for SF1 is the highest whereas SF1 is the highest followed by SF2 and then FCE. For asthenozoospermic sperms progressive motility was found to be highest followed by SF1 and then SF2. Abnormality for SF1 is the highest. In case of asthenozoospermic patients similar across FCE, SF1 and then SF2. Abnormality similar across FCE, SF1 and SF2. Viability for FCE was found to be highest as followed by SF1 and then SF2. Teratozoospermia patients have progressive motility S2 is the highest followed by FCE and then SF1, abnormality for SF1 is the highest and viability is the highest followed by FCE and SF2. Our results indicated statistically significant differences compared to other groups within same condition. (Table 3).

Table 3: Effect of biotin and pentoxifylline on semen samples post thawing for oligozoospermia and teratozoospermia

Semen sample analysis	Normozoospermia (n=15) Pre thaw		Post thaw	Asthenozoospermia(n=8) Pre thaw		Post thaw	Oligozoospermia(n=26) Pre thaw		Post thaw	Teratozoospermia(n=16) Pre thaw		Post thaw	P value
Sperm concentration (10^{-6}/ml)	38×10^{-6}	22×10^{-6}	22×10^{-6}	10×10^{-6}	11×10^{-6}	11×10^{-6}	41×10^{-6}	21×10^{-6}	21×10^{-6}	51×10^{-6}	21×10^{-6}	21×10^{-6}	
Total motility (%)	47.24 ±2.74	95±2.1	84± 1.0	46.67 ±2.57	90±2.1	79± 1.0	35.72 ±1.65	92±2.1	90± 1.0	45.25 ±1.76	92±2.1	88± 1.0	P<0.01
Progressive motility (%)	38.34 ±1.45	40.12 ±2.1	38± 1.5	40.14 ±1.35	38.12 ±2.1	35.0 ±1.5	26.14 ±1.35	38.12 ±2.1	36.0 ±1.5	32.04 ±1.35	38.12 ±2.1	34.0 ±1.5	P<0.01
Abnormality (%)	92±1.8	92±1.8	91± 1.8	95±1.8	94±1.8	93± 1.8	94±1.2	94±1.8	92± 1.8	98±1.2	94±1.8	90± 1.8	P<0.01

Normozoospermic subjects-15; subjects with abnormal semen parameters n=8; The abnormal group included oligozoospermic (n=26), asthenozoospermic (n=8), and teratozoospermia (n=16). One sample t–test with Wilcoxon t-test, P<0.01. The analysis of semen samples was categorized into four groups based on the type of sperm abnormality (normozoospermia, oligozoospermia, and teratozoospermia) and revealed the following results. The parameters measured included sperm concentration, total motility, and progressive motility which was found to increase post-thaw, except for a slight decrease or remain unchanged post-thaw with a significant decrease in teratozoospermia. These findings indicate that cryopreservation generally improves motility while having varying effects on sperm concentration and abnormality percentages. The significant changes were supported by statistical tests, suggesting the observed results (Table 3) (Figure 4).

3.2.7 Eosin and Nigrosin staining in morphology defect sperms

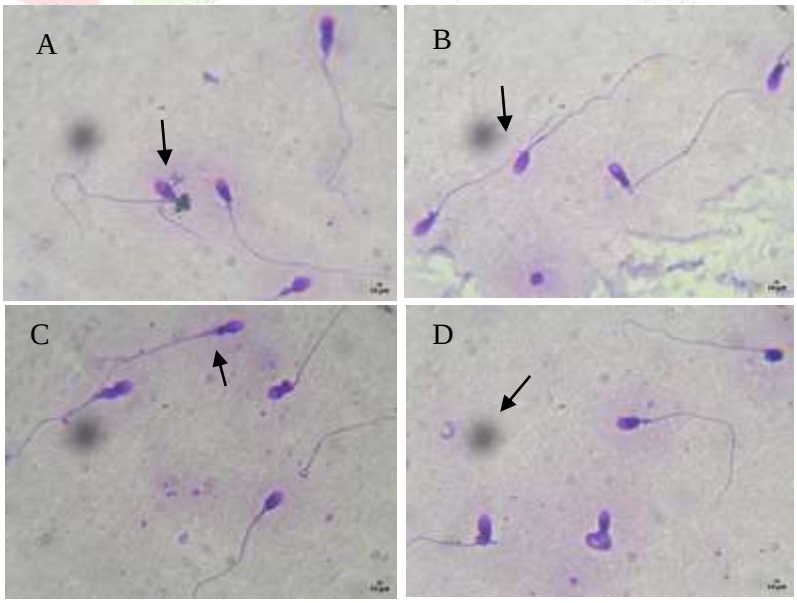

Figure 4: A. Photomicrographs of morphologically defective sperm sample. A. Multiple defects in sperm samples, irregularity in sperm morphological features, B. Head defect observed in sperm samples. C. Neck and midpiece defects observed under 100 X magnification.

3.2.8 Assessment of Enzymatic using Catalase, MDA and Superoxide dismutase level

Mean ± SD value of MDA levels concentration after the spectrometric test in seminal plasma and spermatozoa samples of asthenozoospermic groups were significantly greater compared with the normozoospermic group (Fig.5) (A) A significant correlation was observed between the MDA level of SP and SPZ sample. Additionally, SOD enzymatic activity at 5, 150 U/mL, was deficient to SOD 100U/mL treated groups concerning control significant difference between SOD at 50 and 150 U/mL to other factors. The enzymatic superoxide activity at 100(U/mL) treated semen plasma for the control group was significant ($P<0.05$) and differed between groups. The antioxidant property exhibited significantly ($P<0.05$) higher catalase activity (Fig.5) (B), TAC, SOD (Fig.7) (C), and SOD (100 U/mL) concentration treated sample than the control group. It was recorded from the experimental data that the addition of superoxide molecules at the concentration level of 100U/mL to the diluted spermatozoa sample showed significant improvement in the quality and after-cryopreservation properties. Reduced level of MDA, helps in the protection of spermatozoa samples after supplementation of tocopherol as an additive antioxidant (Figure 5).

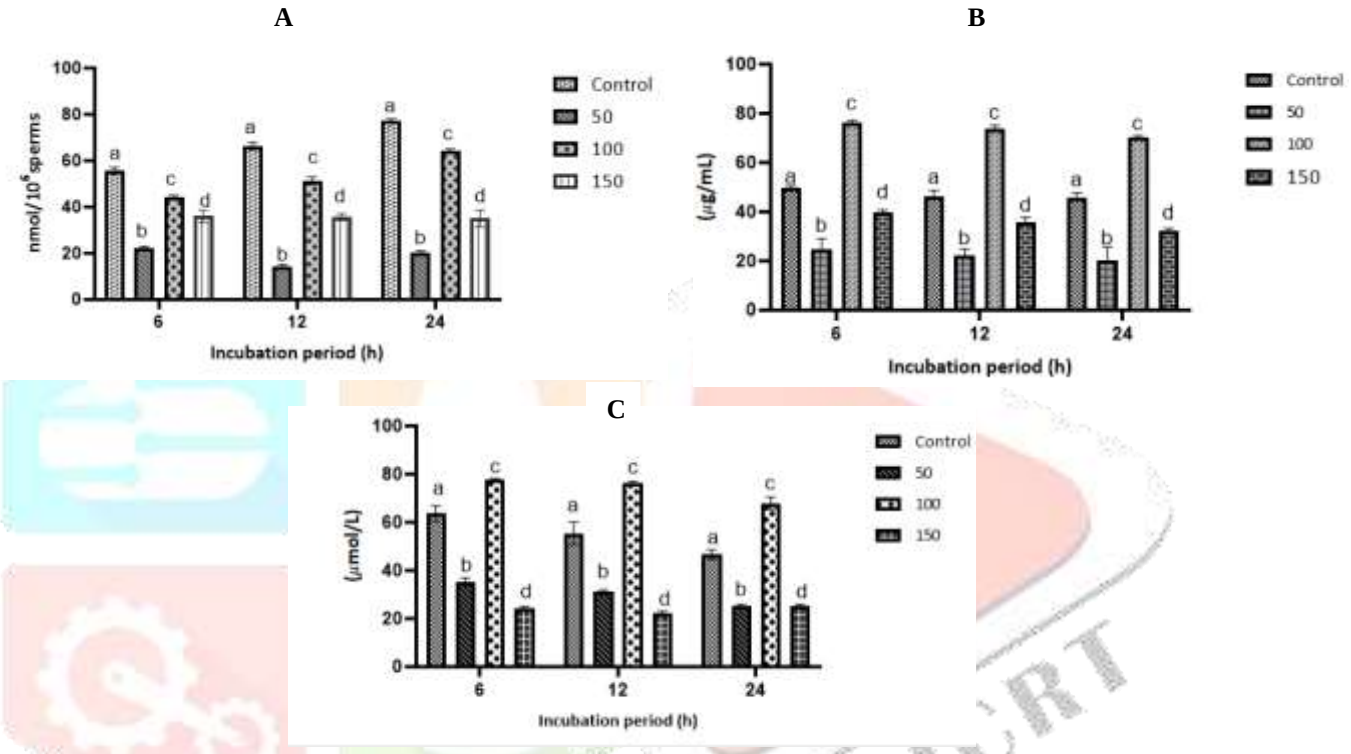

Figure. 5. (A) Malondialdehyde level production semen containing additive at different incubation periods ($p<0.05$) (B) Catalase activity concerning the control group containing semen samples at different incubation periods expressed (µg/mL) indicates ($P<0.05$) (C) Superoxide dismutase activity (µmol/L) concerning the control group containing semen samples at different incubation periods expressed (µg/mL) indicates ($P<0.05$), (a,b,c,d) differ significantly ($P<0.05$).

Discussion: Previous findings have indicated the impact of biotin and pentoxifylline on post-thaw semen samples focusing on sperm samples, sperm abnormalities and motility. Biotin and pentoxifylline concentrations were determined for optimal sperm motility and the study found differences in sperm motility based on treatment concentrations and time intervals. Higher concentrations generally led to higher sperm viability as indicated by data on sperm motility, abnormality, and viability among different groups (Kalthur ,2012). Evaluating the impact of biotin and pentoxifylline on semen samples post-thawing, specifically focusing on sperm abnormalities and motility is a real challenge in the study. It is now becoming more evident that oxidative sperm DNA breakage is a significant factor in male fertility and the ineffectiveness of Assisted reproductive technology(ART).(Fatma Atig,2013). In this study we determined the impact of biotin and pentoxifylline on semen samples post thawing focusing on sperm abnormalities and motility. There is a significant correlation between the FCE and split fractions into normal and pathological semen samples as well as sperm DNA integrity within the sperm ejaculate fractions. When humans ejaculate their spermatozoa are thought to be immobile and mechanically confined in the seminal vesicle's seminogelins, which form the semen coagulum (Lundwall,2002). However, SF1 is high in acid phosphatase, citric acid , magnesium, and zinc in addition to a wide range of proteins (Arver , 1982). According to reports, the prostatic secretion's prostate-specific antigen contributes to the breakdown of semenogelins (Lilja,1989) and has previously been shown to positively correlate with sperm motility

(Elzanaty,2002). The spermatozoa concentration in SF1 and SF2 was slightly higher than in FCE which may be partially explained by this qualitative and quantitative variation in SF1 and SF2 composition. In addition to being in terms of viability, motility, and sperm head shape it was also demonstrated that SF1 had improved sperm DNA integrity. Apart from this superiority in motility, viability, and head shape were also shown to have better DNA integrity. Flagellar motility is influenced by cyclic adenosine 3′,5′ monophosphate, calcium, and protein phosphorylation.(J S Tash,1983). The inability to sustain a high level of cAMP in the cytoplasm of the frozen thawed spermatozoa may be the cause of the marginal drop in motility that was seen in the pentoxifylline group with an increase in incubation time. In the present study, we found the impact of biotin and pentoxifylline on semen samples post thawing focusing on sperm abnormalities and motility. Results have shown improvements in total motility post-thawing with varying effects on perm concentration and abnormality percentages. Higher concentrations of the treatments generally led to higher sperm viability. The precise mechanism of action of biotin is difficult to decipher from these first data. Nevertheless, we tried to determine whether the indirect approach impact is carried out phosphodiesterase enzyme inhibition. The addition of phosphodiesterase IV at several concentrations to the sperm solution obtained using the swim-up technique decreased the motility of the sperm. Biotin-responsive inborn errors in lipid metabolism might result in reduced activity of the biotin-dependent enzymes which also modifies lipid metabolism. It is commonly known that biotin offers nutritional value. Previous studies have demonstrated that a shortage of biotin can result in teratogenesis in humans. (Karl Peter, 2018) as well as in lower mammals. Biotin-responsive inborn errors of lipid metabolism. It is now abundantly evident that biotin plays a critical role in heme production (Atmana, 2007). DNA damage prevention and chromatin structure regulation (Kuroishi, 2011). According to Zempleni 2011, the decrease in biotinylated histones leads to chromosomal instability which is thought to be the origin of the teratogenic effect of biotin deficiency in humans and mice. It was discovered that when the mothers received multivitamin supplements the incidence of birth abnormalities decreased including biotin. The data unequivocally highlights the dietary significance of biotin for fetal development (Cydney, 1978). These data unequivocally highlight the dietary significance of biotin for fetal development or pregnancy. But up until today, its impact on sperm function augmentation remained unknown. However molecular biomarker studies on these parameters are yet to be studied.

III. CONCLUSION

Our study highlights that biotin and pentoxyfillin-supplemented sperm preparation medium may greatly increase post-thaw sperm motility and their endurance in the case of normozoospermia, asthenozoospermia, teratozoospermia, and oligozoospermia sperm preparation medium may greatly increase post-thaw sperm motility and their endurance in the absence of sperm or developing embryos. Because of this biotin and pentoxyfillin combination might be a secure and useful substitute medium to improve the quality of frozen sperm in an ART system. Hence our split fraction study in various ejaculates has revealed differences in the integrity of sperm DNA that have not been previously reported. Therefore, if DNA integrity is a concern using SF-1 instead of the entire ejaculate for assisted conception may be preferable from a therapeutic standpoint.

ACKNOWLEDGMENT

The authors are thankful to REVA University for the contributions to carry out this study.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

FUNDING

This study received no extramural funding.

AUTHOR'S CONTRIBUTION

Manjula Kannasandra Ramaiah provided the Conceptualization did the methodology and the investigation was done and Ashwini Lekkalapudi Somashekar. Intellectual content was given by writing, review, and editing were done by edited and reviewed the paper by Manjula Kannasandra Ramaiah., and Ramesh Nijalingappa have done Citations and References.

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