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A Study of the durability of sperm cell motility and deoxyribo-nucleic acid damage during cryopreservation

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Abstract

Semen cryopreservation is an essential technique in artificial insemination (AI) and assisted reproductive technology (ART). The addition of antioxidants to the freezing medium is a promising strategy to reduce cryo-induced damage to sperm cells. While sperm freezing has been extensively studied, this research aimed to improve the post-thaw durability and functionality of sperm. A total of 110 semen samples 55 from fertile and 55 from infertile individuals were collected and processed. After centrifugation, each sample was mixed with a cryoprotectant medium at a 1:1 (v/v) ratio. Three different cryoprotectant formulations (S1, S2, and S3), each containing varying concentrations of protective agents, were evaluated. The samples were then frozen in liquid nitrogen at −196°C. Post-thaw analyses included assessments of sperm count, motility, vitality, morphology, DNA fragmentation, and reactive oxygen species (ROS) levels representing a major strength of the study due to the comprehensive evaluation of sperm quality. The results showed significant differences in DNA fragmentation between fresh and cryopreserved samples. Among the tested formulations, S3 supplemented with citric acid anhydrous and taurine produced the best outcomes. It significantly improved sperm motility and vitality, while effectively reducing oxidative stress and cryodamage. Statistical analysis using one-way ANOVA and Tukey's HSD test revealed significant differences in sperm morphology between fresh and post-thaw S3-treated samples ($p < 0.05$), with a 95% confidence level. A limitation of the study was the long interval between the use of fresh control samples and the analysis of cryopreserved samples, which may influence comparative accuracy. Despite this limitation, the findings suggest that the S3 formulation is highly effective in preserving sperm quality. These results support its potential integration into fertility preservation protocols in ART. Furthermore, the outcomes may guide clinical practice and support the development of national policies aimed at improving access to advanced cryopreservation techniques as part of comprehensive reproductive healthcare services. *(Afr J Reprod Health 2025; 29 [10]: 30-39).*

Keywords: Antioxidants; ROS; ART; Cryoinjury; DNA damage; Cryoprotectant

Résumé

La cryoconservation du sperme est une technique essentielle en insémination artificielle (IA) et en procréation médicalement assistée (PMA). L'ajout d'antioxydants au milieu de congélation est une stratégie prometteuse pour réduire les dommages causés aux spermatozoïdes par la cryoconservation. Bien que la congélation du sperme ait fait l'objet de nombreuses études, cette recherche visait à améliorer la durabilité et la fonctionnalité du sperme après décongélation. Au total, 110 échantillons de sperme, dont 55 provenant de personnes fertiles et 55 d'individus infertiles, ont été collectés et traités. Après centrifugation, chaque échantillon a été mélangé à un milieu cryoprotecteur dans un rapport 1:1 (v/v). Trois formulations cryoprotectrices différentes (S1, S2 et S3), chacune contenant des concentrations variables d'agents protecteurs, ont été évaluées. Les échantillons ont ensuite été congelés dans de l'azote liquide à −196 °C. Les analyses post-décongélation comprenaient des évaluations du nombre, de la motilité, de la vitalité, de la morphologie, de la fragmentation de l'ADN et des concentrations d'espèces réactives de l'oxygène (ERO) des spermatozoïdes, ce qui représente un atout majeur de l'étude grâce à l'évaluation exhaustive de la qualité du sperme. Les résultats ont montré des différences significatives de fragmentation de l'ADN entre les échantillons frais et cryoconservés. Parmi les formulations testées, la formule S3 supplémentée en acide citrique anhydre et en taurine a donné les meilleurs résultats. Elle a significativement amélioré la motilité et la vitalité des spermatozoïdes, tout en réduisant efficacement le stress oxydatif et les dommages cryogéniques. L'analyse statistique par ANOVA à un facteur et test HSD de Tukey a révélé des différences significatives dans la morphologie des spermatozoïdes entre les échantillons frais et les échantillons traités au S3 après décongélation ($p < 0,05$), avec un intervalle de confiance de 95 %. Une limite de l'étude était le long intervalle entre l'utilisation d'échantillons témoins frais et l'analyse d'échantillons cryoconservés, ce qui pourrait influencer la précision comparative.Malgré cette limite, les résultats suggèrent que la

formulation S3 est très efficace pour préserver la qualité du sperme. Ces résultats plaident en faveur de son intégration potentielle aux protocoles de préservation de la fertilité en procréation médicalement assistée (AMP). De plus, ces résultats pourraient orienter la pratique clinique et soutenir l'élaboration de politiques nationales visant à améliorer l'accès aux techniques avancées de cryoconservation dans le cadre de services complets de santé reproductive. *(Afr J Reprod Health 2025; 29 [10]: 30-39).*

Mots-clés: : Antioxydants; ROS; ART; Cryoblessures; Dommages à l'ADN; Cryoprotecteur.

Introduction

In recent years, cryopreservation techniques have evolved to address the challenges of oxidative stress, DNA damage, and post-thaw sperm viability. Antioxidant-enriched cryoprotectants, such as those incorporating taurine, citric acid anhydrous, and trehalose, have demonstrated superior sperm preservation by reducing reactive oxygen species (ROS) and maintaining sperm integrity. Studies have also explored nanotechnology-based cryoprotectants and low-toxicity vitrification methods, improving post-thaw survival rates while minimizing cryoinjury.

Human semen cryopreservation is a cornerstone of assisted reproductive technology (ART), providing long-term storage solutions for individuals undergoing fertility preservation, sperm donation, and ART procedures such as in vitro fertilization (IVF). This process, which involves the preservation of semen at extremely low temperatures (-196°C in liquid nitrogen), effectively suspends sperm cell activity, making it invaluable in reproductive medicine.[1] However, cryopreservation is not without its limitations. Cryoinjury, induced by oxidative stress, osmotic imbalances, and intracellular ice formation during freezing and thawing, poses significant challenges to maintaining post-thaw sperm quality. These factors adversely affect crucial sperm parameters such as motility, viability, and DNA integrity, thereby reducing the efficacy of ART procedures.

Artificial insemination (AI) has been a key reproductive technique widely used in humans and animals.[2] Studies dating back to the 1970s identified glycerol and dimethyl sulfoxide (DMSO) as commonly used cryoprotectants.[3] More recent research highlights the pivotal role of glycerol-based cryoprotectants in preserving sperm motility and vitality and avoiding cryogenic injury during freezing.[4,5] Maintaining an optimal pH of 7.0–8.0, similar to fresh semen, is a complex and challenging task during cryopreservation. Many studies have reported using cryoprotectants formulated at slightly acidic pH levels, typically between 6.4 and 6.8, to improve preservation outcomes.[6] Buffers incorporated into semen cryoprotectants are critical in balancing pH during freezing and thawing, stabilizing sperm metabolism, and mitigating osmotic shock. Combining glycerol with non-toxic agents like trehalose has shown promise in reducing the required concentration of glycerol, thereby minimizing its toxic effects while maintaining cryoprotective efficacy.

Tris-based cryoprotectants, formulated with Tris(hydroxymethyl) aminomethane, are gaining interest due to their biocompatibility and reduced toxicity compared to conventional cryoprotectants. These buffers effectively stabilize pH during freezing, ensuring the preservation of sperm motility and DNA integrity.[7] Antioxidants have emerged as vital supplements in cryopreservation media. They neutralize free radicals, highly reactive molecules with unpaired electrons, which otherwise damage cellular structures. Vitamins, herbal extracts such as green tea and aloe vera, and sugars like fructose are frequently incorporated into cryoprotectants. Fructose, in particular, is recognized as a practical energy source, improving sperm motility, viability, and acrosome integrity.[8] Herbal antioxidants help combat oxidative stress and enhance cell viability by neutralizing free radicals generated during the freeze-thaw process.[9,10] Tea polyphenol (T. Arjuna bark extract) was used as an antioxidant to protect spermatozoa against lipid peroxidation during cryopreservation in smokers.[26]

Despite significant advancements in cryopreservation techniques, the challenges in developing cryoprotectants that are effective at reduced concentrations while maintaining their protective properties are pressing. In India, the need to identify optimal cryoprotectants that enhance and maintain sperm longevity is urgent. Additionally, cryopreserved semen is indispensable for ART

applications, including IVF and intracytoplasmic sperm injection (ICSI), where high-quality sperm samples are imperative for success. This study, which delves into the effects of various concentrations of cryoprotectant on human semen parameters, is of paramount importance. It focuses on cost efficiency and investigates the impact of cryoprotectants on sperm motility, viability, DNA integrity, and oxidative stress compared to commercially available products. This research aims to significantly contribute to the optimization of semen cryopreservation protocols and their application in modern ART, a crucial step in advancing reproductive medicine.

Methods

Inclusion and exclusion criteria for the study population

To ensure unbiased results and minimize variability, sample randomization was achieved through a systematic selection process. The selection criteria for participants in the current study are based on an age range of 20 to 45 years. The study includes both healthy men and those with reproductive challenges, such as low sperm count, reduced motility, or abnormal morphology, who live in urban areas and are occupationally exposed to air pollution. Participants who are currently using supplements or other medications for infertility are excluded. Additionally, individuals with leucocytospermia (elevated white blood cell counts in semen), sexually transmitted infections, alcohol consumption, or a history of smoking are excluded due to the increased levels of seminal reactive oxygen species (ROS), which reduce antioxidant activity and contribute to diminished motility and abnormal morphology. Each sample was assigned a unique identification code to eliminate any investigator bias during processing and analysis.

Institutional ethical approval number

Ethical approval from the VIT, Vellore (Ref No: VIT/IECH/XI/2022/06) and the Sri Narayani Hospital and Research Centre (SNHRC) Ethical Board (Ref IEC No: 36/04/02/23). All participants were given a written consent form to confirm that they were willing to provide samples for the study, and they were advised that the reports would only be used for academic research purposes.

Sample collection

Human sperm samples were provided by patients undergoing sperm analysis at the Reproductive Medicine Department of SNHRC, Thirumalaikodi, Vellore, Tamil Nadu, India. Patients voluntarily provided semen samples after filling out the consent form. Following three days of abstinence, couples masturbated to collect their semen in a sterile plastic container with the volunteer's name, identification number, date, and time of collection. Number of volunteers who participated in the research, One hundred ten human semen samples were collected, and initial reports were recorded before preservation. The samples were then categorized into fertile (n = 55) and infertile (n = 55).

Sample analysis

After sample collection, the semen container was allowed to liquefy at 37°C for 30 min, initial semen processing involved microscopic analysis using a Makler Counting Chamber. The semen volume was measured with a graduated measuring cylinder. All semen parameters (total motility, progressive motility, and total count) were measured.[11]

Sperm morphology analysis

A thorough examination of sperm morphology was carried out by developing a uniform smear of semen on a slide and staining it with Hematoxylin and Eosin (H&E).[12,13] To perform Haematoxylin and Eosin (H&E) staining, prepare a semen smear on a glass slide and fix it with 95% ethanol for 5–10 minutes. Stain the slide with hematoxylin solution for 3–5 minutes, followed by washing in tap water for 5–10 minutes. Then, dip the slide in eosin Y solution for 30 seconds to 1 minute, rinse with distilled water, and dehydrate with (70%, 95%, 100%) ethanol for 2 minutes each. Rinse the slide in xylene for 3–5 min, add a mounting medium, and place a cover slip. Finally, examine the slide under a microscope using an oil immersion lens (100X).

Preparation of cryoprotectant

Cryoprotectants are made, followed by previous literature using lower-concentration compounds supplied by HIMEDIA.[14] Gentamicin (15,000 IU) was added to avoid semen contamination and limit protein synthesis by any microbes.

Table 1: The different compositions of medium (S1, S2, and S3)

Composition	Units	S1	S2	S3
Glycerol	%	15	10	5
DMSO	%	6	4	2
Tris(hydroxymethyl)aminomethane	G	0.10	0.05	0.01
Citric acid Anhydrous	G	2.0	1.5	0.5
Fructose	G	2.0	2.0	1.0
Sodium Citrate Dihydrate	G	1.5	1.5	0.5
Trehalose	G	1.5	1.0	0.2
pH		7.2	7.2	7.0
Gentamicin	IU	15000	15000	15000
Taurine	G	1.0	0.5	0.2
Make up to	**mL**	**100 mL**	**100 mL**	**100 mL**

Furthermore, 0.2% trehalose was used in all cryoprotectants because of its high water retention capacity. Table 1 shows the chemical composition of each cryoprotectant. Each cryoprotectant was made in a 100 mL volume and aliquoted into various cryotubes/vials (2 mL). The cryotubes/vials were named S1, S2, and S3, and each contained an equal volume of semen and cryoprotectants. Rapid cryopreservation was performed following the protocol with slight changes. A mixed an equal volume of semen with cryoprotectant, mix gently, and incubate it at 4°C for 10-15 min. Place the vials 20 cm above -196°C liquid nitrogen for 15 min and immerse them in the tanker. Two replicates were performed to check the quality and stability of the sperm cells and cryoprotectants. The thawing procedure involved putting the cryopreserved vials in water at normal temperature for 5 min to avoid osmotic shock. They were then kept at room temperature for further analysis.

DNA fragmentation analysis

To perform AO/EtBr staining for DNA fragmentation, cells were washed with cold PBS, and the pellet was resuspended in PBS at the desired concentration. Stock solutions of Acridine Orange (AO) and Ethidium Bromide (EtBr) were prepared in PBS, and equal volumes were mixed to create a working staining solution. This solution was added to the cell suspension, mixed gently, and incubated for a few minutes at room temperature. A drop of the stained cells was placed on a microscope slide, covered with a coverslip, and observed under a fluorescence microscope using appropriate filters for green (AO) and red (EtBr) fluorescence. The apoptotic cells are displayed in bright green.

Nitro-blue tetrazolium (NBT) staining test

The Nitro-blue Tetrazolium (NBT) assay measures the level of reactive oxygen species (ROS) produced by sperm. NBT is a chemical that turns blue when it comes into contact with superoxide radicals. A mixture of 0.1 mL of sample, 0.1 mL of 0.2% nitro-blue tetrazolium (NBT) in saline, and 0.1 mL of phosphate-buffered saline is incubated at 37 °C for 15 min and then left at room temperature for another 15 min. Smears are prepared on a glass slide, fixed with methanol for 3 min, and stained with Pappenheim's stain for 3–5 min. At least 200 individual sperm cells were examined by a microscope.

Statistical analysis

All statistical analyses were done in GraphPad Prism for Windows (GraphPad Software, La Jolla, California, USA). One-way analysis of variance (ANOVA) was used to describe the differences in semen parameters between fresh and cryopreserved groups. The significance is $p < 0.05$. The descriptive statistics data are represented as Mean, Standard deviation, and standard error (±) of the mean (SEM) statistical significance.

Results

Once the semen samples were collected through masturbation, the fresh samples were analyzed for basic semen parameters according to WHO (2021) guidelines. The samples were then loaded in Cryotubes/vials with different concentrations of cryoprotectants and incubated for 187 days at -196ºC liquid nitrogen.

Table 2: Comparison of Infertile semen parameters (fresh, S1, S2 and S3)

Group Parameters	**Fertile Samples (Control)**	**S1**	**S2**	**S3**
Cell Count Millions/mL)	81.89 ± 0.15	1.65 ± 0.19****	14.11 ± 0.20**	35.11 ± 0.15
Vitality	77.80 ± 0.12	14.44 ± 0.11***	29.13 ± 0.28**	72.42 ± 0.15
Prog. Motility	60.36 ± 0.06	12.58 ± 0.14****	16.13 ± 0.12***	58.89 ± 0.13*
Morphology	4.22 ± 0.02	1.05 ± 0.03****	1.84 ± 0.02**	3.93 ± 0.03*

Table 3: Comparison of Infertile semen parameters (fresh, S1, S2 and S3)

Groups Parameters	**Fresh Infertile Samples (Control)**	**S1**	**S2**	**S3**
Sperm Cell Count (Millions/mL)	8.55 ± 0.06	1.07 ± 0.04****	2.49 ± 0.04**	6.79 ± 0.04
Vitality (%)	69.49 ± 0.16	10.25 ± 0.08****	12.93 ± 0.21***	67.58 ± 0.21*
Progressive Motility (%)	55.38 ± 0.12	6.35 ± 0.06****	11.15 ± 0.11**	52.87 ± 0.11
Morphology (%)	4.04 ± 0.02	0.49 ± 0.01***	1.44 ± 0.01*	2.70 ± 0.03

After preservation, sperm cell count, vitality, progressive motility, and morphology were analyzed (Tables 2 and 3).

The values are shown here Mean ± standard error of the mean (SEM); **** indicates extremely significant differences ($p < 0.0001$) across groups. *** and ** denote ($p < 0.001$), whereas * represents ($p < 0.05$) at a 95% confidence level.

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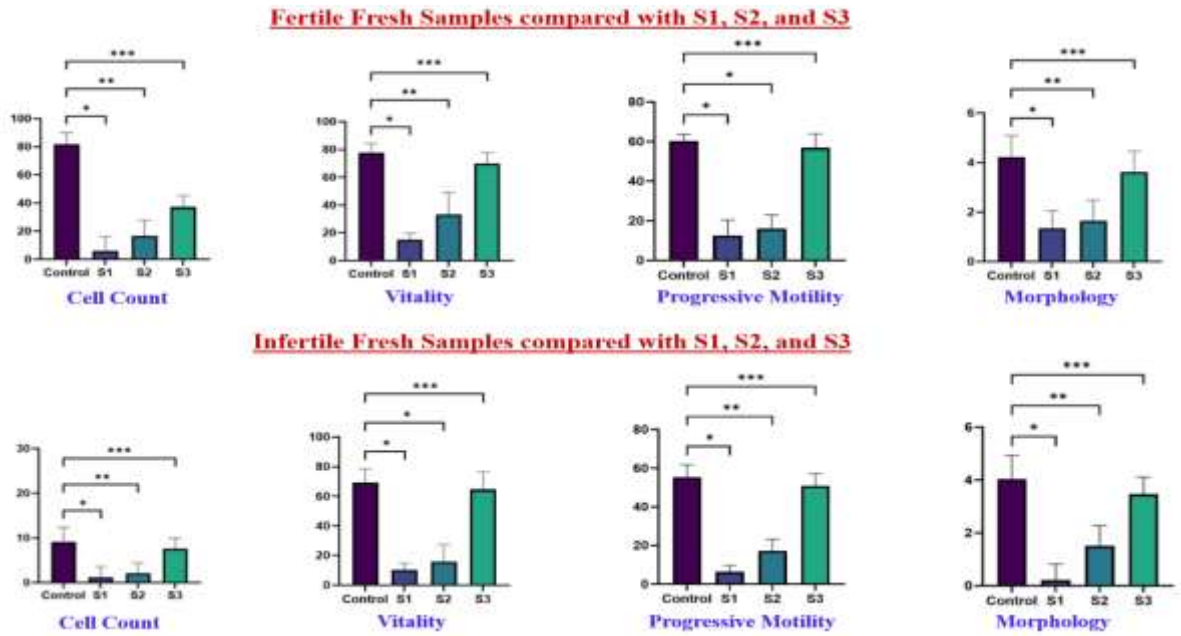
The sperm parameters of fertile and infertile samples in the S3 cryoprotectant, which contains antioxidants, yielded acceptable outcomes. Because it is aliquoted, the number of sperm in the S3 cryoprotectant (35.11 ± 0.15 million/mL) was almost the same as the number of sperm in the control sample (81.89 ± 0.15 million/mL).

In contrast, the glycerol supplement S1 showed a reduction in sperm count (1.65 ± 0.19), vitality (14.44 ± 0.11), progressive motility (12.58 ± 0.14), and increased morphological damage (1.05 ± 0.03) during cryopreservation.

Additionally, the characteristics of infertile semen parameters varied among cryopreserved samples. The sperm cell count in the control group for the infertile sample (S1) was 1.07 ± 0.04 million/mL. In comparison, the S3 cryoprotectants showed 6.79 ± 0.04 million/mL, which is very close to the fresh infertile cell count of 8.55 ± 0.06 after aliquoting the samples. The GraphPad one-way ANOVA was used to check the significant differences between the groups after post-thaw treatment. The outcome of the data represents the significant differences between the groups, as shown in Figure 1. The S3 cryoprotectant with antioxidants improved semen parameters compared to the S1 and S2 cryoprotectant media. The absence of antioxidants was associated with sperm morphology damage, reduced motility, and increased cell death.

The statistical analysis revealed varying levels of significance in sperm parameters when comparing fresh control samples with those subjected to freezing and various treatments. The S3 medium demonstrated superior performance, showing improvements in sperm count, progressive motility, sperm vitality, and sperm morphology compared to S1 and S2.



S3 outperforms S1 and S2 in terms of sperm viability, motility, and morphology in both fertile and infertile samples. Statistical significance markers highlight the reliability of these differences: * designates $p < 0.05$ (statistically significant), ** sy mbolizes $p < 0.01$ (very significant), and *** indicates $p < 0.001$

Figure 1: Comparison and significant differences of semen parameters of fertile and infertile semen samples between the fresh and cryopreserved groups

These results highlight the enhanced effectiveness of the S3 treatment in improving various sperm quality parameters when cryopreserved.

Hematoxylin and Eosin (H&E) analysis

Almost 2-4% of the sperm cell morphology defects (as shown in **Figure 2C**) were observed by using Hematoxylin and Eosin (H&E) stain after cryopreservation in all the vials. Studies revealed that below 3% of sperm morphological defects are acceptable for IVF. In contrast, more than 15-20% of normal morphology is considered the best for treatment.

DNA fragmentation detection assay

AO, a membrane-permeable dye, enters both live and apoptotic cells, binding to double-stranded DNA (dsDNA) and single-stranded RNA (ssRNA). When bound to dsDNA, AO fluoresces bright green, while apoptotic cells with fragmented DNA show an intensified green fluorescence due to increased binding sites. Spermatozoa with an intensified green color are identified as sperm cells with DNA fragmentation. Figure **2A**. Below 10% of DNA damage was observed in the S3 medium and is considered a good sample, whereas more than 80% of DNA damage was observed in the S1 medium.

ROS detection assay

The NBT assay provides a semi-quantitative assessment of ROS production in sperm, making it a valuable tool for evaluating oxidative stress, sperm dysfunction, and potential male infertility. Higher ROS levels indicate oxidative damage, which can impair sperm motility, DNA integrity, and fertilization potential. Cells undergoing an oxidative burst form dark blue formazan deposits at the affected site. As shown in **Figure 2B**, we observed very few sperm cells forming formazan deposits in Control and S3. In contrast, S1 and S2 formed formazan, indicating the presence of reactive oxygen species (ROS) generated during the cryopreservation process. Major oxidative stress was generated in the S1 and S2 medium when compared to the S3 medium.

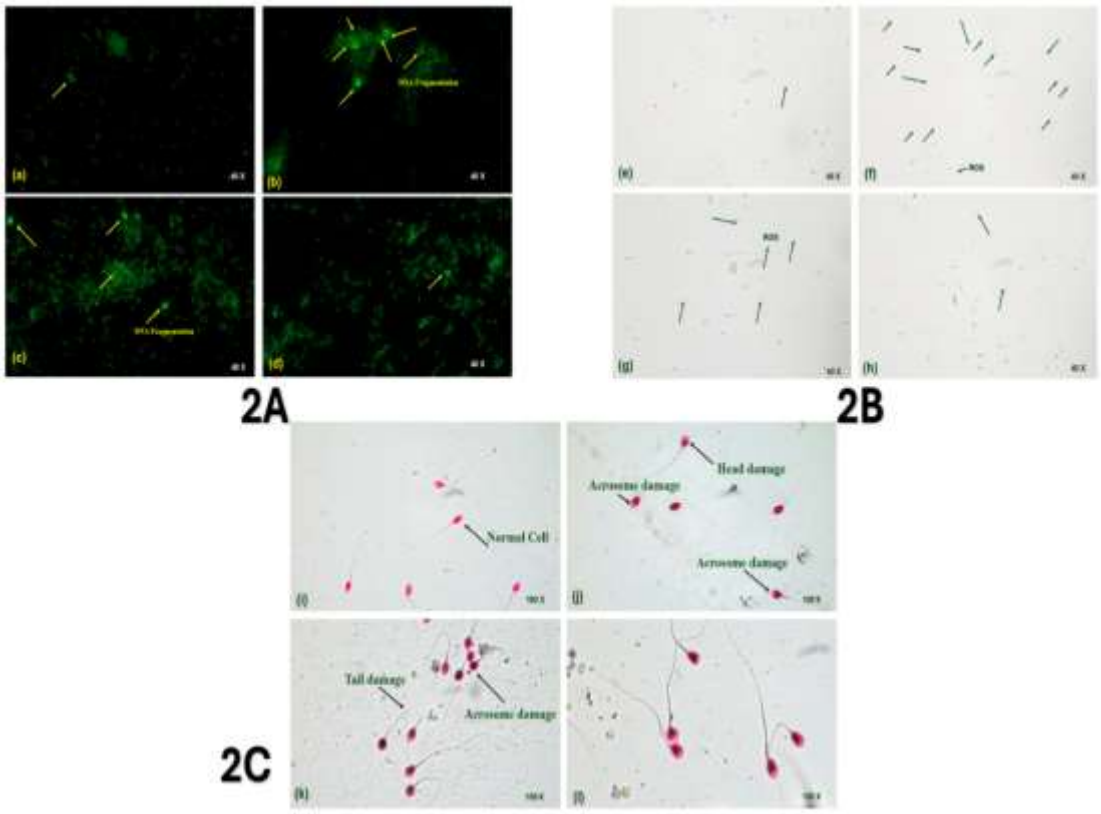


Figure 2A: The DNA fragmentation test was observed after the cryopreservation (a) control, (b) S1 group: Fully fragmented DNA is observed, (c) S2 group: Partially fragmented DNA is observed (d) S3 group: Less DNA fragmentation was observed compared to S1 and S2 medium.

Figure 2B: The arrow mark shows the ROS generation as per µg formazan 10^6 (e) <10 % ROS was observed control sample (f) > 60 % ROS was observed in S1 medium (g) > 30-40 % ROS was observed S2 sample (h) < 15 % ROS was observed control sample.

Figure 2C: Morphological differences were observed under Light microscopy (hematoxylin and eosin staining) (i) normal Sperm Cell in the control sample (j) Acrosome and Head Damage in S1 medium (k) Minor Acrosome damage and tail damage were observed in S2 medium (l) There is no morphological damage was observed in S3 medium.

Discussion

Infertility poses a significant challenge to human evolution, with contributing factors including environmental influences, dietary habits, age, hormonal imbalances, and sexually transmitted diseases.[15,16] The biological differences between fertile and infertile subjects emphasize the critical role of oxidative stress, membrane integrity, and molecular stability in male fertility. These findings highlight the need for targeted therapeutic interventions, optimized cryopreservation techniques, and improved ART protocols to enhance reproductive success for individuals facing infertility.

Cryopreservation of fertile samples in both humans and animals has emerged as an effective tool for fertility preservation. However, the absence of antioxidants creates oxidative stress on sperm cells during freezing and thawing, leading to a decline in semen parameters.[17] Glycerol was used as a cryoprotectant in 1953, marking a pivotal advancement in cryopreservation techniques.[18] Glycerol, at concentrations of 5–10%, prevents cryoinjury by maintaining osmotic balance within cells, reducing osmotic stress during freezing and thawing.[19,20] Additionally, glycerol stabilizes cellular membranes by preventing phase transitions and lipid bilayer disruptions. [21] Complementary

cryoprotectants, such as fructose and Tris buffer, further enhance sperm viability. Fructose improves sperm functionality during freezing and thawing. At the same time, Tris buffer provides high buffering capacity across a wide pH range, safeguarding cellular integrity against acidic or alkaline fluctuations during the cryopreservation process.[22,23]

A key factor in its effectiveness was the precise pH of 6.8-7.2, optimized to support fertility in assisted reproduction. Citric acid anhydrous was crucial in mitigating oxidative stress-induced damage by scavenging reactive oxygen species (ROS) generated during freezing and thawing. This antioxidative mechanism preserved cellular functionality and mitigated oxidative damage.[24] Moreover, toxic substances and free radicals released during metabolic reactions were effectively neutralized by antioxidant-supplemented cryoprotectants. Incorporating taurine in the S3 cryoprotectant provided additional antioxidative benefits, reducing oxidative stress and improving sperm motility and viability. Gentamicin was included to prevent bacterial contamination, reduce infection risks, and preserve sperm quality. Its efficacy in semen preservation, particularly in boar studies, further validates its application in cryopreservation protocols.[25]

Dimethyl sulfoxide (DMSO), another commonly used cryoprotectant, was employed at a lower concentration of 4% to minimize toxicity. Higher DMSO concentrations (>10%) are known to be toxic. Overall, this study highlights the critical role of antioxidants and optimized cryoprotectant formulations in mitigating oxidative stress and DNA damage during sperm cryopreservation. This work underscores the importance of continued research and refinement of cryopreservation protocols to enhance fertility preservation outcomes. Low and balanced concentrations of these compounds were used to minimize the toxicity and reduce oxidative stress and membrane damage. Also, low concentration reduces the production costs, making the cryoprotectant more accessible.

Study strengths and limitations

The major strength of the study development and testing of the S3 novel cryoprotectant enriched with citric acid anhydrous and taurine, which presents a promising innovation in reducing oxidative stress during cryopreservation. Further comprehensive evaluation of multiple semen parameters after post-thaw provides a well-rounded evaluation. The current study has been conducted in laboratory conditions on a limited number of semen samples to observe the morphological alterations and DNA damage in fertile and infertile semen samples after cryopreservation. Furthermore, limitations related to sample size, especially when stratified by fertility status, and the exclusion of functional fertilization outcomes are acknowledged. These constraints were necessary to maintain focus on core post-thaw sperm quality parameters within the scope and resources of the current investigation.

The potential for standardizing antioxidant-enriched cryoprotectants as a best practice in ART laboratories is shown by the S3 cryoprotectant performance. These results can help guide the creation of clinical sperm banking methods, especially for individuals whose fertility is being preserved before receiving chemotherapy or other therapies. The demonstrated effectiveness of bespoke cryoprotectants such as S3 further emphasizes the significance of regulatory frameworks that encourage creativity and make it easier for new biopreservation methods to be approved

Conclusion

Our approach aimed to balance efficacy with cost-effectiveness, ensuring optimal sperm cell stabilization while minimizing oxidative stress (ROS) and DNA damage. Lower concentrations of chemicals were intentionally used to reduce production costs while maintaining efficiency in preserving sperm quality. This strategic formulation allows for a more accessible and practical cryoprotectant without compromising its protective effects during freezing and thawing. The S3 cryoprotectant enhances sperm survival, motility, and morphology, making it excellent for use in assisted reproductive technologies. Its antioxidants minimize oxidative stress and DNA damage, which improves fertilization and embryo development. The improved DMSO concentration (4%) reduces toxicity, allowing for safer usage in IUI, IVF, and ICSI. Furthermore, its cost-effective

composition makes it an affordable option for reproductive treatments.

Future studies should assess the long-term impact of cryopreserved sperm on fertilization, embryo development, and live birth rates in assisted reproductive technologies (ART). This will provide a deeper understanding of how cryoprotectants influence reproductive success beyond initial post-thaw viability. Advanced techniques like RNA sequencing and proteomics could uncover sperm-specific molecular pathways affected by cryopreservation. Biopolymer-based cryogels or synthetic antifreeze proteins may offer superior protection against cryodamage. Exploring cost-effective, portable storage solutions may also expand access to fertility preservation, particularly in low-resource settings.

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Authors contribution

D. Abilash – Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft; T. B. Sridharan - Conceptualization, Project administration, Resources, Supervision, Writing – review and editing.

Data availability

All data generated or analyzed during this study are included in this published article.

Conflict of interest

The authors declare that there are no competing interests.

Compliance with ethical standards

The Institutional Human Ethical Committee, VIT, Vellore approved the study, Ref No: VIT/IECH/XI/2022/06 & Sri Narayani Hospital and Research Center Ethical Committee, Ref IEC No: 36/04/02/23.

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