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Effect of Cryopreservation on Vital and Morphological Parameters of Spermatozoa: A Laboratory-Based Investigation

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Abstract: Sperm cryopreservation is a crucial procedure in Assisted Reproductive Technology (ART); however, it is known to adversely affect the quality of recovered spermatozoa. This study aimed to evaluate the impact of the freezing and thawing processes on the vital and morphological parameters of spermatozoa. The study included 40 semen samples obtained from men with normal semen parameters attending the Al-Sgaaf Center for Infertility Investigations in Mukalla, Hadhramout, Yemen, between December 2022 and March 2023. Semen samples were analyzed pre-freezing and post-thawing according to the World Health Organization guidelines. The results of the study revealed a highly statistically significant decline ($p < 0.001$) in sperm quality following cryopreservation. The mean total motility decreased remarkably from 63.2% to 50.6%, and sperm vitality dropped from 63.9% to 52.4%. Furthermore, normal sperm morphology was severely affected, with the total percentage of morphologically normal spermatozoa decreasing from 35.8% before freezing to 25.9% after thawing. Regarding specific sperm regions, the normal morphology of the head declined from 23.0% to 12.1%, the midpiece from 45.6% to 41.0%, and the tail from 41.5% to 35.9%. In conclusion, the cryopreservation process causes a substantial deterioration in sperm motility, vitality, and normal morphology, highlighting the urgent need to develop optimized cryopreservation protocols and better cryoprotectants to minimize cellular stress and damage during the freeze-thaw cycle

Keywords: Cryopreservation; Sperm; Morphology, Assisted Reproductive Technology

1. Introduction:

Sperm cryopreservation is a fundamental pillar in Assisted Reproductive Technology (ART) and fertility preservation strategies for oncological and non-oncological purposes. It has demonstrated its critical role in sustaining reproductive care even during global health crises [2], [3]. Although this technique is highly effective and allows for the long-term storage of spermatozoa without drastically compromising overall survival rates [4], the freeze-thaw cycle is inherently associated with significant biological challenges [5].

Recent studies indicate that the cryopreservation process subjects spermatozoa to severe osmotic, thermal, and oxidative stress, leading to a substantial deterioration in sample quality and optimal handling timeframes [6], [7]. This cellular stress directly translates into reduced motility, loss of vitality, and detrimental alterations in sperm morphology [8]. Furthermore, cryopreservation contributes to molecular-level damage, including increased DNA

fragmentation, altered DNA methylation, and impaired chromatin compaction, all of which may adversely affect ART outcomes and offspring health [9].

To mitigate these detrimental effects, continuous scientific efforts are directed toward optimizing freezing protocols and exploring novel cryoprotective additives—including exosomes and other bioactive agents—to minimize cryopreservation-induced cellular injury and improve the quality of recovered spermatozoa [10]. Amid these advancements, the accurate and standardized evaluation of semen parameters pre- and post-cryopreservation remains crucial, particularly when managing cases of impaired spermatogenesis or surgically retrieved sperm [7]. In this context, strictly adhering to the updated guidelines outlined in the sixth edition of the World Health Organization (WHO) manual is essential to ensure the quality and reliability of laboratory evaluations [11].

Given this background, the present study aims to thoroughly evaluate the impact of the freezing and thawing processes on vital sperm parameters (motility and vitality). Specifically, the study highlights the detailed morphological alterations occurring in the distinct regions of the spermatozoon (head, midpiece, and tail). By doing so, this research seeks to provide a deeper understanding of cryopreservation-induced cellular stress, ultimately contributing to the optimization of clinical sperm handling protocols.

2. Materials and Methods

2.1 Study Design and Setting

This laboratory-based investigation with apaired before-and-after design was conducted between December 2022 and March 2023 at the laboratory of the AL-Sgaaf Center for Infertility Investigations in Mukalla, Hadhramout Governorate, Yemen. Semen samples were analyzed before cryopreservation and after thawing to evaluate the changes in sperm motility, vitality, and morphology.

2.2 Study Samples and Criteria

The study included 40 men with normal semen parameters who attended the center for routine semen analysis. Inclusion criteria comprised participants with semen characteristics within normal references ranges according to WHO guidelines, including normal sperm concentration and good motility. Individuals diagnosed with severe oligozoospermia, azoospermia, or necrozoospermia were strictly excluded from the study.

2.3 Ethical Considerations

The study protocol was reviewed and officially approved by the Faculty of Science at Hadhramout University. Furthermore, informed consent was obtained from all participants prior to semen collection after explaining the purpose of the research.

2.4 Laboratory Procedures and Cryopreservation Protocol

Macroscopic and microscopic semen analyses were performed in strict accordance with the World Health Organization guidelines [1]. Sperm vitality was assessed using the one-step Eosin-Nigrosin staining technique (FertiPro, Belgium), where unstained spermatozoa were classified as live, and those showing pink/red coloration were considered dead. For morphological evaluation, smears were stained with Hematoxylin & Eosin (H&E) and assessed under 100x objective magnification.

A. Freezing Procedure:

Following complete liquefaction at room temperature, semen samples were mixed with a commercial cryoprotectant medium (Sperm Freez™, FertiPro, Belgium) at a ratio of 1:0.7 (semen: cryoprotectant). The mixture was left to equilibrate for 10 minutes at room temperature. The cryovials were then placed horizontally in a liquid nitrogen vapor bath for at least 15 minutes before being rapidly plunged into liquid nitrogen and stored at -196°C.

B. Thawing Procedure:

The cryovials were retrieved from the liquid nitrogen and immediately thawed in an incubator at 37°C. To remove the cryoprotectant, a specific culture medium (FertiCult™ Flushing Medium, FertiPro, Belgium) was added, followed by centrifugation at 500 × g for 10 minutes. The supernatant

was discarded, and the sperm pellet was diluted with the flushing medium to the appropriate volume for the final post-thaw evaluation.

2.5 Statistical Analysis

Data analyses were performed using SPSS software (version 22.0, IBM Corp., Armonk, NY). A Paired t-test was utilized to compare the mean values of sperm parameters before freezing and after thawing. A p-value of less than 0.05 was considered statistically significant.

Table 1. Distribution of samples according to semen volume

Semen Volume (ml)	Frequency	Percentage %
1.5	5	12.5%
2.0	6	15%
2.2	1	2.5%
2.5	6	15%
3.0	15	37.5%
4.0	4	10%
5.0	1	2.5%
6.0	2	5%
Total	40	100.0%

3. Results

3.1. Macroscopic and Baseline Semen Characteristics

Macroscopic evaluation in Figure (1) and Table (1) revealed that the majority of semen samples (67.5%) underwent complete liquefaction within 30 minutes, while the remaining samples required a longer time. Semen volumes ranged from 1.5 and 6.0 ml, with the highest proportion of samples (37.5%) having a volume of 3.0 ml. In addition, all samples exhibited normal macroscopic features, including a homogeneous appearance, a white-gray opaque color, normal viscosity, and a pH ranging from 7.2 to 8.0. Microscopic examination showed that 45.0% of the samples had a sperm concentration between 15 and 30 million spermatozoa/ ml, indicating that the majority of participants had semen parameters within the normal reference range.

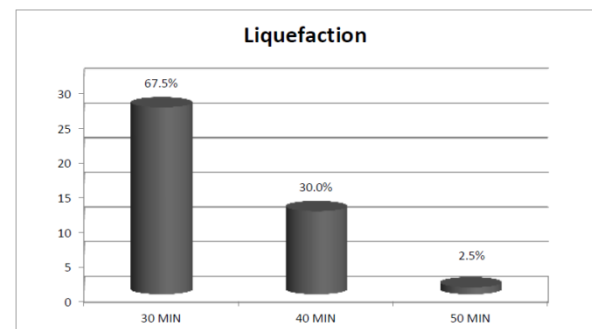


Figure 1. The samples according to semen liquefaction

3.2. Effect of Cryopreservation on Sperm Motility

The results of cryopreservation in the Table (2) revealed a significant reduction in total sperm motility. The mean total motility decreased from 63.2% before freezing to 50.6% after thawing. Statistical analysis confirmed that this decline was highly significant ($p < 0.001$).

3.3. Effect of Cryopreservation on Sperm Vitality

The findings represented in the Table (2) indicated a remarkable decline in sperm vitality following the freeze-thaw cycle. The mean vitality percentage dropped from 63.9% pre-freezing to 52.4% post-thawing, representing a highly statistically significant difference ($p < 0.001$).

3.4. Effect of Cryopreservation on Sperm Morphology

The results in the Table (2) demonstrated a substantial deterioration in the percentage of morphologically normal spermatozoa after cryopreservation. The mean value of overall normal morphology significantly decreased from 35.8% before freezing to 25.9% after thawing ($p < 0.001$). Further morphological evaluation of specific sperm regions (head, midpiece, and tail) revealed varying degrees of deterioration. The percentage of normal head morphology sharply declined from 23.0% pre-freezing to 12.1% post-thawing. The normal midpiece morphology decreased from 45.6% to 41.0%. The normal tail morphology reduced from 41.5% to 35.9%. All the morphological changes following cryopreservation were highly statistically significant ($p < 0.001$).

Table 2. The effects of cryopreservation on sperm motility, vitality, and morphology

Semen parameters	Before cryopreservation		After cryopreservation		Mean Reduction	P-value
	Mean $\pm$ S.D.	C.V.	Mean $\pm$ S.D.	C.V.		
Total Motility	63.2 $\pm$ 9.2	14.6	50.6 $\pm$ 7.5	14.7	12.6	0.001
Vitality	63.9 $\pm$ 5.2	8.2	52.4 $\pm$ 4.9	9.3	11.5	0.001
Normal Morphology	35.8 $\pm$ 3.6	10.1	25.9 $\pm$ 4.4	17.0	9.9	0.001
Head	23.0 $\pm$ 5.4	23.4	12.1 $\pm$ 4.3	35.2	10.9	0.001
Midpiece	45.6 $\pm$ 4.4	8.6	41.0 $\pm$ 3.5	9.7	4.6	0.001
Tail	41.5 $\pm$ 4.1	9.7	35.9 $\pm$ 3.5	9.9	5.6	0.001

*C.V. = Coefficient of Variation

Mean Reduction = Mean before cryopreservation – Mean after cryopreservation

4. Discussion

Sperm cryopreservation is an indispensable procedure for fertility management within Assisted Reproductive Technology (ART) [2], [7]. However, the findings of the current study demonstrated that the freeze-thaw cycle induces a highly statistically significant deterioration ($p < 0.001$) in all vital and morphological parameters of spermatozoa. These results are consistent with recent scientific consensus emphasizing the profound sensitivity of human spermatozoa to the physical and chemical stressors associated with cryopreservation [5], [12].

Our study revealed a drastic decline in the mean total motility (from 63.2% to 50.6%) and vitality (from 63.9% to 52.4%) post-thawing. From a biological perspective, this functional impairment can be attributed to the thermal and osmotic shock cells experience during water efflux and intracellular ice crystal formation. This process often leads to plasma membrane disruption and a loss of selective permeability [8]. Furthermore, the observed decline in vitality may be associated with mechanisms reported in previous studies, including oxidative stress and membrane damage induced during freezing and thawing study [13], [14]. However, these biological mechanisms were not directly assessed in the present study.

Morphologically, our results indicated a severe deterioration in the overall percentage of normal sperm (from 35.8% to 25.9%). Notably, the cryopreservation process exerted a devastating impact on the sperm head region, where the percentage of normal head morphology plummeted from 23.0% to 12.1%. Recent literature elucidates that the mechanical stress inflicted by ice crystals leads to acrosome damage and aberrant chromatin condensation within the sperm head [16]. Previous studies have reported an association between cryopreservation-induced morphological alterations and DNA fragmentation; however, DNA integrity was not evaluated in the current study [9], [16].

It is important to note that the study did not incorporate advanced molecular assessments, such as the direct quantification of sperm DNA fragmentation or intracellular reactive oxygen species (ROS) levels. Therefore, these potential mechanisms were discussed based on established physiological principles rather than direct experimental measurement.

Regarding the midpiece and tail regions, the study also recorded a significant decline in their normal morphological forms. This deformation is primarily ascribed to mitochondrial damage in the midpiece caused by oxidative stress. Such damage impairs ATP production, providing a mechanistic explanation for the acute loss of sperm motility observed in current study [6], [17].

Methodologically, this study was strictly aligned with the updated criteria of the sixth edition of the WHO laboratory manual [1]. This alignment grants our findings high reliability and effectively eliminates laboratory bias [11], [18]. In light of these findings, it is evident that current cryopreservation protocols, despite their clinical utility, still inflict substantial ultrastructural damage to spermatozoa [19]. This underscores the persistent need to refine slow-freezing techniques and explore the efficacy of supplementing freezing media with potent antioxidants (such as Coenzyme Q10 or innovative sugars) to mitigate oxidative stress and preserve the morphological integrity of human sperm [10], [20].

5. Conclusion

This study clearly demonstrates that sperm cryopreservation, despite its critical role in assisted reproductive technology (ART), significantly compromises semen quality. We recorded a marked decline in total motility and vitality, alongside severe deterioration in overall normal

morphology, with the sperm "head" region being particularly susceptible to freeze-thaw damage. These findings underscore the urgent need to develop innovative cryopreservation protocols and explore the supplementation of freezing media with advanced antioxidants. Such improvements are vital to minimize cellular stress, enhance the quality of recovered spermatozoa, and ultimately improve ART success rates.

6. Limitations of the study

Despite the significance of the findings, this study has certain limitations. First, the sample size was relatively small (40 participants), and data collection was restricted to a single medical center (Al-Sgaaf Center in Mukalla), as it was the only accessible facility available for conducting the analyses during the study period. This may limit the broad generalizability of the results. Second, sperm motility was evaluated as total motility only, while progressive and non-progressive motility categories were not separately analyzed. Furthermore, oxidative stress markers and sperm DNA fragmentation were not measured; therefore, the biological mechanisms underlying the observed deterioration could not be directly confirmed. Third, the present study was designed as a paired before-and-after observational study, in which each semen sample served as its own control. Sperm parameters were evaluated before cryopreservation and subsequently reassessed after thawing, allowing direct comparison within the same sample while minimizing inter-individual variability. Therefore, a separate control group was not considered necessary for the primary objective of assessing the impact of the freeze-thaw process on sperm quality. Nevertheless, the absence of an independent control group is acknowledged as a methodological limitation.

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تأثير الحفظ بالتجميد على الخصائص الحيوية والظاهرية للحيوانات المنوية: دراسة استقصائية مختبرية

عبد الرحمن سالم ياسين، محمد سالم الحداد، خالد صالح بن الشيخ أبوبكر

المخلص: يُعد الحفظ بالتجميد للحيوانات المنوية إجراءً بالغ الأهمية في تقنيات الإنجاب المساعدة (ART)، ولكن من المعروف أن هذه العملية تؤثر سلباً على جودة الحيوانات المنوية المستردة. هدفت هذه الدراسة إلى تقييم تأثير عمليتي التجميد والذوبان على العوامل الحيوية والشكلية للحيوانات المنوية. شملت الدراسة 40 عينة سائل منوي لرجال راجعوا مركز السقاف لأبحاث العقم في المكلا والذين أظهرت تحاليلهم مؤشرات سائل منوي ضمن الحدود الطبيعية، خلال الفترة من ديسمبر 2022 إلى مارس 2023. تم تحليل العينات قبل التجميد وبعد فك التجميد وفقاً لمعايير منظمة الصحة العالمية. أظهرت النتائج انخفاضاً ذا دلالة إحصائية عالية ($p < 0.001$) في جودة الحيوانات المنوية بعد فك التجميد؛ حيث تراجع المتوسط الكلي للحركة بشكل ملحوظ من 63.2% إلى 50.6%، وانخفضت نسبة الحيوية من 63.9% إلى 52.4%. علاوة على ذلك، تأثر الشكل الطبيعي للحيوانات المنوية بشدة، إذ انخفضت النسبة الكلية للشكل السليم من 35.8% قبل التجميد إلى 25.9% بعده. وفيما يخص التقييم المورفولوجي لأجزاء الحيوان المنوي، انخفضت نسبة الشكل الطبيعي للرأس من 23.0% إلى 12.1%، وللقطعة الوسطى من 45.6% إلى 41.0%، وللذيل من 41.5% إلى 35.9%. يُستنتج من الدراسة أن عملية الحفظ بالتجميد تسبب تدهوراً كبيراً في الحركة والحيوية والشكل الطبيعي للحيوانات المنوية، مما يُسلط الضوء على الحاجة الماسة لتطوير بروتوكولات تجميد ومواد حماية أفضل لتقليل الإجهاد والتلف الخلوي أثناء دورة التجميد والذوبان.

الكلمات الدالة: الحفظ بالتبريد، الحيوانات المنوية، حيوية الحيوانات المنوية، حركة الحيوانات المنوية، شكل الحيوان المنوي، تقنيات الإنجاب المساعدة.