ECRONICON OPEN ACCESS

EC GYNAECOLOGY
Research Article

Evaluation of Three Different Sperm Cryopreservation Media for Human Sperm Freezing and Thawing Compared to Traditional TEST-Yolk Buffer

Sangjoon Lee[1], Isabelle Mason[1,2], Evelina Manvelyan[1,2], Kathryn Coyne[1,2], Joseph Findley[1,2], Rachel Weinerman[1,2], Rebecca Flyckt[1,2] and Sung Tae Kim[1,2]*

[1]*University Hospitals Fertility Center, Beachwood, Ohio, USA*

[2]*Case Western Reserve University, School of Medicine, Cleveland, Ohio, USA*

***Corresponding Author:** Sung Tae Kim, Associate Professor, Department of Reproductive Biology, CWRU School of Medicine and Lab Director, Division of Reproductive Endocrinology and Infertility, University Hospitals Fertility Center, Ohio, USA.

Received: January 27, 2026; **Published:** February 09, 2026

Abstract

Introduction and Aim: Sperm cryopreservation is widely used for male fertility preservation and fertility treatment via assisted reproductive technology (ART). Various sperm cryopreservation media are commercially available. The aim of this study is to estimate the post-freeze motility drop using three commercially available sperm cryopreservation media in comparison to that after the traditional sperm freeze using TEST-Yolk Buffer (TYB).

Methodology: Semen samples that met the inclusion criteria were obtained. Samples were divided into four equal aliquots and each aliquot cryopreserved using different sperm cryopreservation media (A, B, C and control TYB) according to each vendor's protocol. Motility was evaluated before and after freeze-thaw process. Statistical analyses were performed using ANOVA and paired t-test.

Results: Average sperm motility prior to cryopreservation was 60.2%. As expected, all four sperm cryopreservation media were associated with a drop in sperm motility ($p < 0.001$). Control group, TYB showed significantly higher post-thaw motility (35.7%) compared to study groups A, B, and C (26.3%, 24.2% and 25.5% respectively). There was no significant difference in post-thaw motility drop among the experimental groups A, B and C.

Conclusion: TYB use is generally discouraged due to utilization of animal products and theoretical possibility of viral contamination. Nonetheless TYB is more affordable and results in better post-thaw sperm motility compared to newer human sperm cryopreservation media. In order to promote and further encourage the use of these second generation sperm cryopreservation media, further research and improvements to the media have to take place to have at least comparable but preferably superior post-thaw sperm survival.

Keywords: *Sperm Cryopreservation Media; TEST-Yolk Buffer; Sperm Freezing; Sperm Thawing*

Introduction

Semen cryopreservation has become a cornerstone of assisted reproductive technology (ART) and male fertility preservation. This technique is used prior to a patient undergoing cytotoxic cancer treatment, before a genital surgical procedure, or when the patient is unable to provide a fresh semen sample for an ART procedure [1-3].

However, the process of cryopreservation puts sperm at risk of cryodamage both during the freezing process as well as the thaw.

During the freezing, damage is observed via alterations of the crucial plasma membrane, due to extreme temperature changes as well as osmotic stress [4]. The freezing of solution means that extracellular solutes are no longer dissolved in solvent, and thus water from inside the cell moves outside, thereby dehydrating and damaging intracellular proteins [5]. As the cell thaws, further damage can occur due to reactive oxygen species (ROS). These ROS can come from surrounding leukocytes, nearby damaged sperm, or within the cell's own mitochondria [5]. The increase in ROS causes DNA damage as well as decrease in sperm function (e.g. motility), which affect the viability of the sperm [4-8]. Because of the sperm damage that occurs during cryopreservation, multiple collections or extraction procedures would need to be performed if optimal cryopreservation techniques are not developed. Thus, a significant amount of research has gone into determining these optimal settings and techniques for cryopreservation to maximize sample survival.

One such area of significant study is optimizing the cryopreservative media in which the sperm is frozen. There are multiple components to media, which vary from product to product. The basic backbone consists of a cryoprotective agent, an energy source and a pH buffer; some media also add antioxidants, physiological salts and antibiotics.

The addition of cryoprotectants has been shown to decrease plasma membrane damage by decreasing cytoplasmic hyperosmolarity that would occur with freezing, as well as prevent intracellular and extracellular ice formation [5,8]. Cryoprotectants are low-molecular weight substances used to minimize stress caused by freezing and thawing. Cryoprotective agents can fall into two categories: permeating and non-permeating substances. Permeating cryoprotectants (e.g. glycerol) work by creating an osmotic gradient, allowing water to leave and the cells to shrink. They also can lower the freezing point of the media and provide intracellular protection. The non-permeating cryoprotectants (e.g. disaccharides, albumin) are too large to cross the cell membrane and therefore act by pulling water out of the cells. Both permeating and non-permeating agents also decrease the freezing point, keeping the extracellular environment in liquid phase and thereby minimizing ice crystal damage. Sugars are present in most media, providing a myriad of cryoprotective effects. Glucose and fructose can act as an energy source for the sperm during cryopreservation, while sucrose is the most common sugar used as a non-permeable cryoprotectant [9].

Since a significant portion of sperm and tissue damage occurs from reactive oxygen species (ROS), many media will have antioxidants added. These antioxidants can be enzymatic (e.g. glutathione peroxidase or catalase) or non-enzymatic (e.g. radical scavengers such as vitamins E or C, taurine, selenium or zinc) [5,8].

Some cryopreservation media will also contain antifreeze proteins/glycoproteins, which are biologic anti-freezing agents that decrease the freezing point of the solution, thereby inhibiting ice crystal formation and stabilizing phospholipids and unsaturated fatty acids [8].

The process of performing semen collection in both human and animal subjects is rife with opportunity for infection. This contamination can not only lead to the dissemination of pathogens, but the microorganisms themselves can decrease overall semen quality [10]. Thus, many media will also contain an antibiotic (e.g. gentamicin) in order to decrease risk of contamination and infection.

While the need for cryoprotectant is widely accepted and the base components are similar (glycerol, energy source, pH buffer), there are multiple commercially available media with little data to support which is the most effective [5,11,12]. One of the most broadly accepted media is test-yolk buffer (TYB), a mixture of buffer solution, egg yolk, glycerol, and antibiotic, as it has been frequently studied and multiple times displayed acceptable post-thaw sperm quality [6].

While studies have analyzed the differential effects of commercially available on sperm viability during sperm freezing and thawing [12,13], we sought to build upon these studies by comparing commercially available cryopreservation media with each other and with TYB.

Materials and Methods

Semen samples were obtained from 10 patients at a single fertility center from October 2023 to February 2024. Inclusion criteria included: volume > 1.5 mL; concentration > 17 million/mL; motility > 20%. Semen analysis was performed according to WHO criteria 2010 [11]. After liquefaction for 30 minutes at 37°C, samples were frozen and thawed according to each cryopreservation media's protocol (Figure 1 and table 1). The cooling rate and the thawing procedure were performed according to the instructions of the manufacturer of each cryopreservation media.

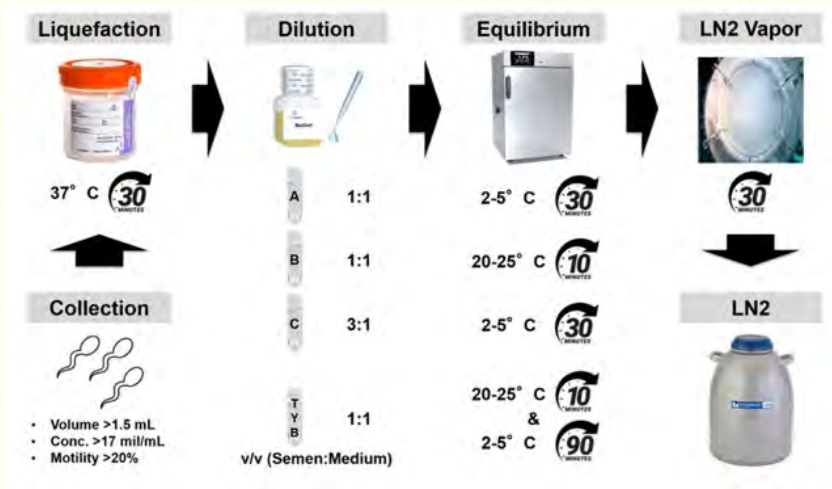

Figure 1: *Overall experimental scheme and comparison of protocols.*

Cryo Media	Dilution* v/v (Semen:Medium)	Equilibrium (20-25°C)	Equilibrium (2-5°C)	Liquid nitrogen Vapor
A	1:1	3 minutes	30 minutes	30 minutes
B	1:1	10 minutes	-	
C	3:1	-	90 minutes	
TYB	1:1	10 minutes	90 minutes	

Table 1: *Brief summary of sperm freezing protocol.*

**Add drop-wise, slowly, over a 30-second period.*

All samples were equally divided into four aliquots. Each of the four cryopreservation media was used to cryopreserve one aliquot of each sample. Briefly, after liquefaction for 30 minutes, sperm count was performed, and the semen were diluted into 1:1 or 3:1 (v/v) with each cryopreservation media. The diluted semen was loaded into cryo-tubes and the mixture was left at room temperature for a minimum of ten minutes or 2 - 5°C for 10 minutes according to the manufacturer's recommendations. Cryo-tubes were attached to a cane and then suspended above the surface of the liquid nitrogen (LN2) for the same period. Finally, we stored cryo-tubes at LN2 tank until thawing them.

The cryopreserved aliquots from all groups were thawed by manufacturer's protocol. The cryo-tubes were removed from LN2 tank and incubated at 37°C for 30 minutes. Then, sperm concentration was assessed using a disposable counting chamber designed for accurate analysis of semen samples.

Motility was evaluated before and after freeze-thaw process. Statistical analyses were performed using ANOVA and paired t-test.

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board of University Hospitals (STUDY20201371). Informed consent was obtained from all subjects involved in the study.

Results

The average percentage of motile sperm prior to cryopreservation was 60.2% (range: 20.0% to 87.0%). All four sperm cryopreservation media were associated with decreased sperm motility ($p < 0.001$). Control group, TYB, showed significantly higher post-thaw motility (35.7%) compared to study groups A (26.3%), B (24.2%), and C (25.5%) ($p = 0.02$ control versus group A, $p = 0.004$ control versus group B, $p = 0.006$ control versus group C respectively) (Figure 2). There was no significant difference in post-thaw motility drop among the experimental groups A, B and C. The average reduction of post-thaw motility in TYB was -24.5% compared to -33.9% in study group A, -36.0% in study group B, and -34.7% in study group C that TYB group showed significantly less reduction rate of post-thaw sperm motility ($p < 0.001$) (Table 2).

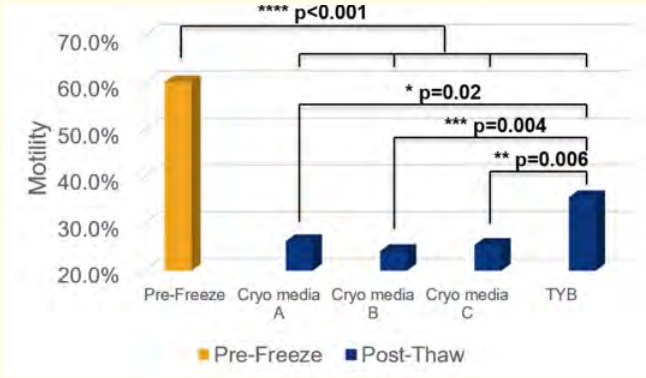

Figure 2: *Post-thaw sperm motility comparison of different cryo media compared to traditional TEST-yolk buffer.*

Cryo Media	Average motility reduction	Standard deviation	p value (compared to TYB)
A	-33.9%	0.1561	p = 0.01997
B	-36.0%	0.1594	p = 0.00356
C	-34.7%	0.1660	p = 0.00637
TYB	-24.5%	0.1293	N/A

Table 2: *Average motility reduction of each cryo media after thawing sperm.*

Discussion

This study shows that the choice of cryopreservation media can have a significant difference on the post-thaw motility of sperm, and that TYB media is superior to the other media. As with all previous cryopreservation studies, we saw a drop in overall sperm quality (motility) between the fresh and frozen samples, which further emphasizes the importance of utilizing fresh semen samples when possible in ART [12,14]. However, for patients undergoing cryopreservation for future use, optimizing the cryopreservation process is essential.

Raad., *et al*. compared various cryopreservation media and found significant differences in survival, coiled tails and DNA fragmentation between different media, with no media proving superior [12].

Fabozzi., *et al*. compared TYB with another common media, Sperm Freeze, and found TYB to be superior in terms of post thaw viability and motility [13]. Given both TYB and Sperm Freeze contain similar penetrating cryoprotectant (glycerol), they surmised that it was the non-penetrating egg yolk that explained the improved results [13]. The egg yolk is composed of phospholipid-rich low-density lipoproteins that maintain a gel-like composition during freezing.

Within the samples of our own study, TYB utilized egg yolk as the non-penetrating cryoprotective agent, while the other three used human serum albumin, which was similar to the Sperm Freeze used in Fabozzi's experiment, while the glycerol remained constant across all media. Therefore, this study adds to the growing body of literature suggesting that egg yolk is a superior form of non-permeating cryoprotectant.

Reactive oxygen species can be catastrophic for sperm and tissue survival due to damage of the mitochondria and creation of self-perpetuating peroxidase damage [5]. They can also bind to and damage proteins within the cell and cause and efflux of cholesterol from the sperm, thereby increasing membrane fluidity [5]. However, within our study, only Media C contained any antioxidant, and it did not prove superior to its counterparts. The TYB does contain cholesterol, thereby providing supplemental cholesterol in the inevitable ROS-driven efflux, thus stabilizing the cellular membrane. While it did not contain any additional antioxidant, there are antioxidants that exist in seminal plasma, which could be contributing to the lack of ROS-induced damage given our samples were not washed prior to cryopreservation [14]. Furthermore, Media C did not prove to be superior to either Media A or B, neither of which contained an antioxidant. A systematic study looking at various antioxidants both alone and in combination within human samples are needed to best delineate which antioxidant, or combination, is the most effective in sperm cryopreservation.

Media C also contained a non-ionic surfactant that was not present in the other medias studied. The role of this surfactant is to protect against fluid mechanical damage by acting as a stabilizing agent [15]. While studies have shown that this surfactant decreases air-liquid interface damage, most of those were done in insect and plant cryopreservation studies, not in human sperm studies [15]. In our study, it did not yield a statistically significant improvement in post-thaw motility compared to any of the other media.

Sucrose is the most commonly used non-permeable cryoprotective sugar, however, multiple studies have found that trehalose is a superior sugar for protecting sperm in various species [16]. The unique structure of trehalose keeps water away from headgroups during freezing and forms glycosylated bonds, which increases cell viability, maintains cellular integrity, and protects the sperm from damage from cryopreservation, all of which are not possible with other mono- or disaccharides [16]. Gholami., *et al*. compared trehalose and a structurally similar sugar, gentiobiose, on various semen parameters [17]. The group goes on to purport that through improved membrane coating, higher number of hydrogen bonds, fewer glucose-lipid bonds and increased penetration, gentiobiose is even superior to trehalose. This study proves the critical nature that specific components of cryopreservation media can have on the survival of sperm. While Gholami could clearly articulate what aspects of the different sugars lead to their superior function, it is more difficult to do with TYB, whose exact mechanism is unclear [13]. Similarly to how trehalose and gentiobiose can form bonds to stabilize and protect the sperm, the phospholipid-rich LDL of the yolk likely protects the sperm in a superior fashion.

While market costs for all media could not be easily discerned, Media C and TYB were displayed on the company websites, and Media C was 2.6 times more expensive than TYB ($5.31/mL vs $2.04/mL). Media C also had considerably more additives (antioxidants, non-ionic surfactant) than the other media but did not display any improved post-thaw motility, in fact, it was inferior to its more affordable

counterpart, TYB. This further emphasizes the need for standardized, controlled studies within human sperm in order to discern what additives are necessary for effective cryopreservation. Without these studies, companies will continue to market media with more additives at a higher price without any data to prove its superiority.

While TYB was superior in our study and is more affordable compared at least to Media C, it does have its drawbacks. Most significantly, the yolk is an animal byproduct, and while the TYB is marketed as virus-free and irradiated, there is still a risk of contamination with microbial agents [18]. Thus, there has been a sect of cryopreservation research looking into creating animal-free cryopreservation media. Sicchieri., *et al*. created a media composed of salts, carbohydrates, purified proteins, amino acids and synthetic cholesterol, to which they added the antioxidant L-acetyl-carnitine and synthetic soybean phosphatidylcholine (PC) [19]. The PC is comprised of triglycerides, fatty acids, glycolipids, sterols and sphingophospholipids, which allow soybean PC to have similar cryopreservative effects as egg yolk. In their study, they found no statistically significant difference between their media and TYB on post-thaw motility and DNA fragmentation index, creating an exciting future for an animal-free cryopreservation media.

Our study is meaningful in that it provides an updated comparison of sperm motility between major commercially available cryopreservation media. We were limited by the fact that we were able to compare TYB with three other market-available solutions. As previously discussed there are multiple other novel components, such as trehalose, gentiobiose and animal-free media, which are ripe for further comparative research studies.

While some may see only measuring semen motility as a limitation, we believe it is the most critical aspect of the semen analysis, and showing statistically significant differences in motility based on cryopreservation media utilized is extremely valuable. Crucial ART decisions are based off of the semen motility, such as whether to proceed with intrauterine insemination or *in vitro* fertilization. It is also the primary determinant as to whether conventional fertilization can be performed versus intracytoplasmic sperm injection. Our study shows that couples may be unnecessarily funneled into more expensive and invasive procedures due to the cryopreservation media utilized.

Currently, sperm motility is the most reliable method of assessing sperm quality and its reproductive potential, and while performing sperm DNA fragmentation (SDF) assessments is becoming more widely used, it is still not routinely assessed in IVF labs worldwide nor is it recommended in routine semen analysis by the WHO or European Association of Urology (EAU) [20]. We wanted to make our findings as generalizable as possible and thus focused on measurements that are consistently performed in IVF labs worldwide. Also, while understanding whether the differences in semen motility between cryopreservation media could be due to reactive oxygen species and subsequent SDF, the fact that the motility decreased more significantly with certain cryopreservation media is the salient point of the study.

Future studies should be performed to assess whether other aspects of sperm quality (e.g. SDF) change based on cryopreservation media selected, and whether subsets of motility (e.g. slow vs fast vs non progressive) are affected differently by cryopreservation media although previous studies showed no significant difference in SDF between different types of cryoprotectants [12,21]. While TYB is widely available and proved superior in our study, this is also a call to continue the development of yolk-free media as described above, or with sugars such as gentiobiose that may prove to be equally effective without using animal-based media. This is also a call to continue to develop studies to compare media within humans in order to understand whether certain additives actually add to the efficacy of the media or just add to the cost.

Conclusion

In conclusion, TYB is more affordable and results in better post-thaw sperm motility compared to newer human sperm cryopreservation media. In order to promote and further encourage the use of these sperm cryopreservation media that does not contain animal product,

further research and improvements to the media have to take place to have at least comparable but preferably surpass the post-thaw sperm survival.

Disclosure

The authors have nothing to report. This project was presented as a poster at the annual symposium of College of Reproductive Biology in May 2024.

Conflict of Interest

Authors don't have any conflict of interest.

Author's Contribution

Sangjoon Lee and Isabelle Mason contributed equally to this work.

Bibliography

1. S-H Song., *et al*. "Long-term experience of sperm cryopreservation in cancer patients in a single fertility center". *World Journal of Men's Health* 37.2 (2019): 219-225.
2. GM Borate., *et al*. "Cryopreservation of sperm: a review". *Cureus* 14.11 (2022): e31402.
3. E Bosch., *et al*. "The future of cryopreservation in assisted reproductive technologies". *Frontiers in Endocrinology* 11 (2020): 67.
4. S Ozimic., *et al*. "Sperm cryopreservation today: approaches, efficiency, and pitfalls". *Current Issues in Molecular Biology* 45.6 (2023): 4716-4734.
5. A Hungerford., *et al*. "Sperm cryopreservation: current status and future developments". *Reproduction Fertility and Development* 35.3 (2022): 265-281.
6. R Pereira., *et al*. "Major regulatory mechanisms involved in sperm motility". *Asian Journal of Andrology* 19.1 (2017): 5-14.
7. M Nijs., *et al*. "Cryopreservation of human sperm". *Human Fertility* 4.3 (2001): 158-163.
8. M Hezavehei., *et al*. "Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches". *Reproductive BioMedicine Online* 37.3 (2018): 327-339.
9. J Liu., *et al*. "Cryopreservation of human spermatozoa with minimal non-permeable cryoprotectant". *Cryobiology* 73.2 (2016): 162-167.
10. CS Santos., *et al*. "Current and alternative trends in antibacterial agents used in mammalian semen technology". *Animal Reproduction* 17.1 (2020): e20190111.
11. P Stanic., *et al*. "Comparison of protective media and freezing techniques for cryopreservation of human semen". *European Journal of Obstetrics and Gynecology and Reproductive Biology* 91.1 (2000): 65-70.
12. G Raad., *et al*. "Cryopreservation media differentially affect sperm motility, morphology and DNA integrity". *Andrology* 6.6 (2018): 836-845.
13. G Fabozzi., *et al*. "Evaluation of the efficiency of two different freezing media and two different protocols to preserve human spermatozoa from cryoinjury". *International Journal of Reproductive Medicine* (2016): 6059757.

14. F Mahdavinezhad., *et al*. "Protective roles of seminal plasma exosomes and microvesicles during human sperm cryopreservation". *Reproductive BioMedicine Online* 45.2 (2022): 341-353.

15. KC Lowe., *et al*. "Beneficial effects of Pluronic F-68 and artificial oxygen carriers on the post-thaw recovery of cryopreserved plant cells". *Artificial Cells, Blood Substitutes, and Immobilization Biotechnology* 29.4 (2001): 297-316.

16. A Najafi., *et al*. "Trehalose and glycerol have a dose-dependent synergistic effect on the post-thawing quality of ram semen cryopreserved in a soybean lecithin-based extender". *Cryobiology* 66.3 (2013): 275-282.

17. D Gholami., *et al*. "Beneficial effects of trehalose and gentiobiose on human sperm cryopreservation". *PLoS One* 18.4 (2023): e0271210.

18. RS Jeyendran., *et al*. "Cryopreservation of human sperm in a lecithin-supplemented freezing medium". *Fertility and Sterility* 90.4 (2008): 1263-1265.

19. F Sicchieri., *et al*. "Phosphatidylcholine and L-acetyl-carnitine-based freezing medium can replace egg yolk and preserves human sperm function". *Translational Andrology and Urology* 10.1 (2021): 397-407.

20. RB Danis., *et al*. "Sperm morphology: history, challenges, and impact on natural and assisted fertility". *Current Urology Reports* 20.8 (2019): 43.

21. LK Thomson., *et al*. "The DNA integrity of cryopreserved spermatozoa separated for use in assisted reproductive technology is unaffected by the type of cryoprotectant used but is related to the DNA integrity of the fresh separated preparation". *Fertility and Sterility* 92.3 (2009): 991-1001.

Volume 15 Issue 2 February 2026