Use of polyvinylpyrrolidone and hyaluronic acid as sperm slower agent to compare recovery rate, motility rate, and viability of spermatozoa post thawing

Uso de polivinilpirrolidona y ácido hialurónico como agente más lento del esperma para comparar la tasa de recuperación, la tasa de motilidad y la viabilidad de los espermatozoides después de la descongelación

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Resumen

Infertility affects 8-12 percent of couples in their reproductive years, with malefactors accounting for 20-30 percent of cases. Freezing sperm is one technique to avoid ICSI (IntraCytoplasmic Sperm Injection) delays by conserving spermatozoa. PVP and Hyaluronic Acid (HA) slow down the movement of spermatozoa, hence the best spermatozoa have faster motility. There have been no previous studies comparing frozen spermatozoa with PVP and HA exposure. The goal of this study is to examine the recovery rate, motility rate, and viability rate of spermatozoa exposed to PVP versus HA as a handling medium before cryopreservation of individual spermatozoa. The research was carried out in the Medical Biology Laboratory of the Faculty of Medicine, Universitas Airlangga. This study used ejaculate from 10 volunteers as a sample. The WHO technique was used to assess all samples for spermatozoa motility, concentration, and morphology. To make spermatozoa handling easier, about 10-15 motile spermatozoa were selected for cryopreservation in PVP and SpermSlow media using a micromanipulator. Selected spermatozoa were then transferred to a cryotec that had already been pre-filled with sperm freeze media and cryopreserved using the ultra-rapid freezing procedure. Overnight, spermatozoa were frozen in liquid nitrogen. Finally, the quality of post-thawed spermatozoa was evaluated. Finally, the recovery rate, motility rate, and viability rate of post-thawed spermatozoa were evaluated. The recovery rate (p=0,520), motility rate (p=0,595), progressive motility rate (p=0.162), and viability rate (p=0,762) of spermatozoa with and without PVP exposure before low sperm count cryopreservation are all the same in this study. There is no difference between PVP and HA, except that Hyaluronic Acid has a little higher rate of progressive motility, although more testing on the ultrastructure of sperm function is needed.

Keywords: cryopreservation, motility, viability, PVP, hyaluronic acid

Abstract

La infertilidad afecta del 8 al 12 por ciento de las parejas en sus años reproductivos, y los malhechores representan del 20 al 30 por ciento de los casos. La congelación de esperma es una técnica para evitar retrasos en la ICSI al conservar los espermatozoides. La PVP y el ácido hialurónico (HA) ralentizan el movimiento de los espermatozoides, por lo que los mejores espermatozoides tienen una motilidad más rápida. No ha habido estudios previos que comparen espermatozoides congelados con exposición a PVP y HA. El objetivo de este estudio es examinar la tasa de recuperación, la tasa de motilidad y la tasa de viabilidad de los espermatozoides expuestos a PVP frente a HA como medio de manipulación antes de la crioconservación de los espermatozoides individuales. La investigación se llevó a cabo en el Laboratorio de Biología Médica de la Facultad de Medicina, Universitas Airlangga. Este estudio utilizó la eyaculación de 10 voluntarios como muestra. Se utilizó la técnica de la OMS para evaluar la motilidad, concentración y morfología de los espermatozoides en todas las muestras. Para facilitar el manejo de los espermatozoides, se seleccionaron entre 10 y 15 espermatozoides móviles para la crioconservación en medios PVP y SpermSlow utilizando un micromanipulador. Luego, los espermatozoides seleccionados se transfirieron a un cryotec que ya se había llenado previamente con medio de congelación de esperma y se crioconservaron mediante el procedimiento de congelación ultrarrápida. Durante la noche, los espermatozoides se congelaron en nitrógeno líquido. Finalmente, se evaluó la calidad de los espermatozoides post-descongelados. Finalmente, se evaluaron la tasa de recuperación, la tasa de motilidad y la tasa de viabilidad de los espermatozoides posdescongelados. La tasa de recuperación (p=0,520), la tasa de motilidad (p=0,595), la tasa de motilidad progresiva (p=0,162) y la tasa de viabilidad (p=0,762) de los espermatozoides con y sin exposición a PVP antes de la criopreservación con bajo recuento de espermatozoides son todas iguales en este estudio. No hay diferencia entre PVP y HA, excepto que el ácido hialurónico tiene una tasa un poco más alta de motilidad progresiva, aunque se necesitan más pruebas sobre la ultraestructura de la función de los espermatozoides.

Palabras clave: criopreservación, motilidad, viabilidad, PVP, ácido hialurónico

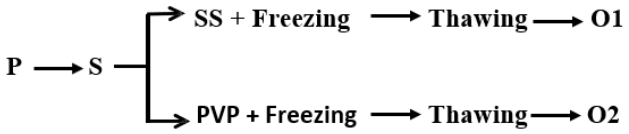
Introduction

Cryopreservation has advanced significantly in assisted reproduction, including techniques for freezing spermatozoa before ICSI to avoid delays when spermatozoa are not discovered during ejaculation[1,2]. Male fertility can be preserved for years by freezing spermatozoa and testicular tissue, regardless of the cause of infertility[3]. Because a single viable spermatozoon is usually adequate for clinical therapies, research into the effects of cryopreservation on these cells has been postponed[4,5]. Previous cryo-injury models are conflicting, and many of the findings are now outdated as new compounds. As a result of new clinical needs and current clinical practice, cryostorage of spermatozoa or testicular tissue is becoming more important: assisted reproduction, preservation of fertility following chemotherapy, radiotherapy, or various surgical procedures, and confirmation of seronegativity for sexually transmitted diseases in semen banking[5,6]. Patients who had azoospermia after oligozoospermia were found to be 52 percent of the time[7]. Sperm from PESA, TESE, and cryopreservation for low-count sperm can be employed in the case of severe oligozoospermia[8,9]. After thawing, the best spermatozoa will produce the best results. The technical can be difficult because the best motility and morphology sperm are hard to come by. Polyvinylpyrrolidone (PVP) and Hyaluronic Acid are two sperm slowing agents that are commonly utilized nowadays. There is an increasing understanding of the risks of slowing sperm mobility with polyvinylpyrrolidone (PVP), a treatment often employed before ICSI. The research described here looks at an alternate product that incorporates hyaluronate, a chemical occurring naturally in the reproductive system, to reduce sperm movement. PVP and hyaluronic acid increase extracellular viscosity[10]. Hyaluronic acid will phagocytize the spermatozoa's head since it has the same components as the Cumulus Oophorus matrix[10,11]. Phagocytosis is the process by which a cell utilizes its plasma membrane to engulf a large particle, leading to an internal compartment named the phagosome[12]. Although there are still numerous issues, there is a growing understanding of potential problems with exposing PVP, and HA is commonly utilized. Their impacts on detailed and integrated fertility parameters, on the other hand, have not been documented. This study will concentrate on the impact of both on male fertility markers. The purpose of this study is to compare the recovery rate, motility rate, and viability rate of spermatozoa exposed to PVP versus HA as a handling medium before individual spermatozoa cryopreservation.

Materials and methods

This study examined the recovery rate, motility, and survival of spermatozoa following cryopreservation and thawing between spermatozoa selected with Hyaluronic acid and sperm selected with Polyvinylpyrrolidone in an experimental study with posttest only research design (PVP). Figure 1 depicts the general course of the research.

Fig. 1 General course of the research



Participants in this study were spermatozoa donors who came to Airlangga University's Medical Biology Department between September and November 2021 and met the following criteria: 18-40 years old, abstinence 2-7 days, volume >1.5 ml, sperm concentration >100 sperm/ml, motility >40 percent, and progressivity >32 percent, agreed to take part in the study, and azoospermia, teratozoospermia, and leukozoospermia samples were taken systematically, according to the research criteria, over a set period until the required number of samples were reached.

Nikon Diaphot 300 inverted microscope with Narishige MM188 micromanipulator, liquid nitrogen tube, hand counter, timer, Eppendorf micropipette, refrigerator, laminar airflow, tweezers, scissors, tube rack, and Styrofoam box are among the study instruments. Fresh human sperm samples, sodium citrate dihydrate, SpermSlowTM (Ref 1094400A, Origio, Denmark), SpermfreezeTM (Ref SSP001, FertiPro, Belgium), sperm wash media, liquid nitrogen, object-glass, cover glass, a yellow disposable tip, Pasteur pipette, 60 mm petri dish, 1.5 ml Eppendorf tube.

Semen samples were produced by masturbation into sterile glass containers and allowed to liquefy for 20-30 minutes. The motility, concentration, and viability of the spermatozoa were assessed after liquefaction using the WHO fifth edition in 2010. The outcomes were written down on the recording sheet.

The tools used for the collection must be clean and sterile, as well as the same temperature as the sperm temperature. Sterilization is done to prevent microorganisms from infecting the sperm and lowering the quality of the sperm. After sterilization, the tools are wrapped in aluminum foil and stored at 37°C until needed[7,12].

The WHO 2010 method was used to examine samples for semen analysis for microscopic and macroscopic inspections. The simple washing approach was used for sperm preparation: The cement is placed in the tube, and the basic washing medium is poured in a 1:1 ratio to the volume of cement. The supernatant was gently removed after centrifuging 300-500 g for 5-10 minutes. Pellets are ready to be selected for spermatozoa[13-15].

Sperm Selection.

PVP and SpermSlow™ were labeled on dishes that had been preheated at 37⁰C for 5 minutes. Drizzled 10 µl of labeled PVP and added 2 µl of spermatozoa suspension to the PVP drops. 5 µl of SpermSlow™ is dripped, 5 µl of culture media is placed next to the SpermSlow™ drop, and 2 µl of spermatozoa suspension is deposited next to the culture medium drop in a labeled SpermSlow™. A connecting bridge was created from the three droplets of SpermSlow™, Culture medium, and Spermatozoa. Using a holding pipette equipped with a micromanipulator, select 10-15 spermatozoa in a petri dish filled with mineral oil. The process of picking the best sperm is carried out under the guidance of an andrologist with years of experience and a license.

Sperm Freezing.

Mix 0.8 ml of HEPES (FertiCult, FertiPro, Belgium) with 0.2 ml of Human Serum Albumin (HSA, Vitrolife, Sweden) and 0.7 ml of SpermFreeze to make the freezing medium (FertiPro, Belgium). Cryotec and straw were labeled, and 2 µl of freezing media was dripped over the cryotec for each treatment. Using a micromanipulator, 10-15 chosen spermatozoa are transported to the cryotec. Allow 3 minutes for the mixture to cool to room temperature. Tweezers should be used to move the cryotec 4 cm above the liquid nitrogen (LN2) for 2 minutes. The cryotec was injected into LN2 after 2 minutes. The cryotec was covered with a straw and kept in a liquid nitrogen tube after 1 minute. The entire cryopreservation process took place in a liquid environment.

Thawing

Check the name and treatment on the straw label for authenticity. Look under a microscope after opening the Cryotec cover straw and placing the Cryotec tip on a slide that has been dripped with a 5µl HEPES medium. After 20 minutes of thawing, count the number of spermatozoa to determine the recovery rate and motility. To test viability, add Eosin and cover with mineral oil.

Statistical Analysis

Quantitative data was evaluated using IBM SPSS Statistics and a comparison test. The Shapiro-Wilk test was used to determine the normality of the data distribution, and the paired T-test was used to compare the results.

Characteristics of sample.
The following are the characteristics of 10 spermatozoa samples that participated in subject to this study:

Table 1. Characteristics of sample

Parameter	Result
Age (years); mean ±SD	31.20±4.63
Volume (ml); mean± SD Consentration (million/ml); mean± SD Progressive Motility (%); mean±SD Non Progressive Motility (%); mean ±SD Immotil (sperm); mean±SD Morphology (%); rerata±SD	2.86 ± 1.50 32.35 ±28.91 51.70±10.41 10.50±7.62 37.80±10,60 1.8±1.13

According to the data acquired, the donors' mean age was 31.20 years, with a standard deviation (SD) of 4.63; this age range reflects the reproductive age of men. The mean ejaculate volume was 2.86 ml, with a standard variation of 1.20 ml. The average concentration of 32.35 million spermatozoa per ml with an SD of 28.91 million was found in all ejaculate samples with a normal volume of > 1.5 ml. Spermatozoa movement was divided into three categories: progressive (51.7%), non-progressive (10.5%), and immotile (37.8%). The normal morphology is 1.8 percent, with a standard deviation of 1.13 percent.

Table 2. Test Results of the Normal Distribution of Research Variables

Variable	P-value	Result
Recovery rate	0,318	Normal
Motility rate	0,873	Normal
Viability Progressivity	0,258 0,952	Normal Normal

Because there were ten samples, the Shapiro Wilk test was used to determine the data's normality. The test results demonstrated a normal data distribution because all values were greater than 0.05.

Table 3. Paired T-Test Results from recovery rate

Variable	PVP Mean ± SD	Hyaluronic acid Mean ± SD	p
Recovery rate	94,00% ± 10,75%	97,00% ± 6,22%	0,520

According to these data, the Hyaluronic acid group has a slightly higher average recovery rate of 3% than PVP. However, there is no statistically significant difference in motility rate between the two groups, with a p-value of 0.520.

Table 4. Paired T-Test Results from motility rate

Variable	PVP Mean ± SD	Hyaluronic acid Mean ± SD	P
Motility rate	57,20% ± 9,28%	53,22% ± 12,50%	0,595

Based on these findings, the PVP group has a slightly higher average motility rate of 3,98% than Hyaluronic acid. However, statistics show no significant difference in motility rate between the two groups, with a p-value of 0.595.

Table 5. Paired T-Test Results from Progresivitas

Variable	PVP Mean ± SD	Hyaluronic acid Mean ± SD	P
Progresivitas	5,00% ± 5,42%	11,20% ± 13,25%	0,162

Based on these findings, the Hyaluronic acid group has a slightly greater average survivability of 6,2 percent than PVP. Nonetheless, statistics demonstrate no significant difference in motility rate between the two groups, with a p-value of 0.162.

Table 6. Paired T-Test Results from viability

Variable	PVP Mean ± SD	Hyaluronic acid Mean ± SD	P
Viability	76,23% ± 9,28%	79,80% ± 10,45%	0,214

Based on these findings, the Hyaluronic acid group has a slightly higher average survivability of 3,57 percent than PVP. However, statistics indicate no significant difference in motility rate between the two groups (p-value = 0.214).

Discussion

The Zona pellucida (ZP) was once a standard method of freezing low sperm counts. Despite this, exposing sperm to the biologic agent of the zona pellucida might lead to genetic or viral contamination. Because Cryotops are non-biological carriers, using them to prevent contamination is a viable option[16,17].

A few topics in the reference research were not covered in detail. Thus, some changes were made based on a preliminary investigation. Due to the difficulty in transferring spermatozoa into Hypo-osmotic solution for Hypo-Osmotic Swelling test, several modifications were made: spermatozoa selection process using two spermatozoa selection medium, selecting 10 of the best spermatozoa before freezing, mixing the freezing medium with spermatozoa for 3 minutes, freezing was carried out using ultra-rapid freezing where the time and distance was 3 cm for 2 minutes, post-viability checking thawing using Due to the difficulty in transferring spermatozoa into a Hypo-osmotic solution for the Hypo-Osmotic Swelling test, Eosin staining was used.

The morphology of the sperm is both the head membrane and tail shape, which will affect the ICSI success rate. Spermatozoa cryopreservation and sperm thawing have a

risk of chromatin damage. The morphology of the sperm is both the head membrane and tail shape, which will affect the ICSI success rate[18,19]. The evaporation of liquid nitrogen prevents submersion after 2.5 minutes. The recovery rate of modest quantities of spermatozoa after cryopreservation was more than 90%, with motility of 32%[20-22].

Although PVP and Hyaluronic acid have a high viscosity that slows spermatozoa motility, there was no significant delay between the two. PVP is thought to cause acrosomal responses and microstructural alterations in the sperm nucleus and spermatozoa membranes. However, no adverse consequences have been detected in infants born after PVP ICSI.[23-28]

Although sperm selection with PVP and HA did not yield meaningful effects, HA did lower miscarriage rates by 18%[13,14]. Although hyaluronic acid improved embryo quality and implementation rate, it did not affect fertility.[24] Between the PVP and Hyaluronic acid groups, there was a substantial difference in DNA integrity.[8] According to research, there was no significant difference in embryonic growth and fertility between the PVP and Hyaluronic acid groups; the priority was the selection of sperm with the best morphology, which would yield superior outcomes[12]. The level of defects in embryo development (1PN or 3PN) at the time of division was more significant in the PVP group after sperm selection with PVP and Hyaluronic acid[19].

Intracellular and extracellular ice crystals, cell dehydration, osmotic stress, and recrystallization following thawing all-cause spermatozoa damage during cryopreservation[9,15]. This entire process can harm the cell membrane's integrity, and if the mitochondria are damaged, motility will be hampered[2,22]. The chromatin, morphology, and membrane integrity of spermatozoa can be damaged by freezing and thawing. Patients who were fertile and had strong DNA integrity had a lower impact on the cryopreservation process when cryoprotectants were used[13]. After freezing, the motility rate did not differ significantly between the PVP and the HA in this investigation.

The vitality of spermatozoa before and after freezing was determined in several ways. No spermatozoa will be frozen immotile due to the utilization of 10-15 spermatozoa that have been selected and have good motility (100 percent motile and viable). The Hypoosmotic Swelling Test (HOST) was used initially for viability testing. However, because spermatozoa transfer takes longer and there is a risk of loss during transfer, the Eosin test was used instead.

Although the difference was not statistically significant, hyaluronic acid showed a 6.2 percent better progressivity movement than PVP. Due to the sample size being too vast, the results of the progressivity movement for both Hyaluronic Acid and PVP had a wide range of results, ranging from good to poor quality sperm. Spermatozoa from low-quality sperm resulted in a 40% reduction in spermatozoa yield compared to spermatozoa from normal sperm. Torres et al. This can be caused by flagella damage or timing problems. Exposure of spermatozoa to PVP can cause ultrastructural damage to flagella, mitochondria, and spermatozoa membrane integrity[26,28]. Another study compared the motility of spermatozoa at 20 and 40 minutes and discovered that the motility of spermatozoa at 40 minutes was higher. If we wait for longer, the spermatozoa may move, but this was not achievable in our investigation because the decline was seen for 20 minutes. The Eosin viability test was continued, and the drop was coated with mineral oil to prevent evaporation.

The bias between research samples is a problem in this study. In this study, normozoospermia to cryptozoospermia were the inclusion criteria. The quality of spermatozoa was found to impact the thawing cryopreservation results in another investigation significantly. Because the contract duration with PVP was limited to less than 10 minutes[21], certain spermatozoa were exposed to PVP for only a few minutes on average. There was no control group in this study. The study's shortcoming is that it only looked at recovery rate, viability, motility, and progression, not DNA fragmentation, chromatin condensation, successful pregnancy, or embryo development. Because of the evaporation that can occur during the thawing process, coating with mineral oil will make it challenging to add the Eosin solution.

Conclusions

After freezing spermatozoa with Hyaluronic acid or PVP, there is no significant change in recovery rate, motility, progressivity, or viability. The outcomes of spermatozoa progression were slightly different in the Hyaluronic acid group, with a difference of 6.9% better than the PVP group. More samples and research into the homogeneity of the quality of the spermatozoa samples, as well as more severe inclusion and exclusion criteria, are needed. PVP and HA cause damage to the ultrastructure of spermatozoa, as evidenced by tests such as DNA fragmentation and chromatin condensation.

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References

1. Hosseini A, Khalili MA, Talebi AR, Agha-Rahimi A, Ghasemi-Esmailabad S, Woodward B, Yari N. Cryopreservation of low number of human spermatozoa; which is better: vapor phase or direct submerging in liquid nitrogen?. Human Fertility. 2018 May 11.https://doi.org/10.1080/14647273.2018.1456681.

2. Beck-Fruchter R, Shalev E, Weiss A. Clinical benefit using sperm hyaluronic acid binding technique in ICSI cycles: a systematic review and meta-analysis. Reproductive biomedicine online. 2016 Mar 1;32(3):286-98.https://doi.org/10.1016/j.rbmo.2015.12.001.

3. Parmegiani L, Cognigni GE, Bernardi S, Troilo E, Ciampaglia W, Filicori M. "Physiologic ICSI": hyaluronic acid (HA) favors selection of spermatozoa without DNA fragmentation and with normal nucleus, resulting in improvement of embryo quality. Fertility and sterility. 2010 Jan 15;93(2):598-604. https://doi.org/10.1016/j.fertnstert.2009.03.033.

4. Bessonnat J, Brouillet S, Sintzel S, Gillois P, Bergues U, Boutte-Busquet C, Thomas-Cadi C, Hennebicq S. In cryptozoospermia or severe oligozoospermia is sperm freezing useful?. Basic and clinical andrology. 2014 Dec;24(1):1-0. https://doi.org/10.1186/2051-4190-24-15.

5. Mortimer D, Björndahl L, Barratt CL, Castilla JA, Menkveld R, Kvist U, Alvarez JG, Haugen TB. A practical guide to basic laboratory andrology. Cambridge University Press; 2022 Feb 28.https://doi.org/10.1017/CBO9780511729942.

6. Endo Y, Fujii Y, Shintani K, Seo M, Motoyama H, Funahashi H. Single spermatozoon freezing using Cryotop. Journal of Mammalian Ova Research. 2011;28(1):47-52.https://doi.org/10.1274/jmor.28.47

7. Parmegiani L, Cognigni GE, Ciampaglia W, Pocognoli P, Marchi F, Filicori M. Efficiency of hyaluronic acid (HA) sperm selection. Journal of assisted reproduction and genetics. 2010 Jan;27(1):13-6.https://doi.org/10.1007/s10815-009-9380-0.

8. NASRESFAHANI MH, RAZAVI S, Mardani M, Aboutorabi R, SHEYKHI A, MOHAMMAD PM. Effect of polyvinyl pyrrolidone on sperm membrane integrity and chromatin status.International Journal of Fertility and Sterility 1 (4): 155–58. https://doi.org/10.22074/ijfs.2007.46208.

9. Fabozzi G, Starita MF, Rega E, Alteri A, Colicchia A, Piscitelli C, Giannini P. 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 Jul 26;2016. https://doi.org/10.1155/2016/6059757.

10. Hossain AM, Osuamkpe CO. Sole use of sucrose in human sperm cryopreservation. Archives of Andrology. 2007 Jan 1;53(2):99-103. https://doi.org/10.1080/01485010701225675.

11. AbdelHafez FF, Desai N, Abou-Setta AM, Falcone T, Goldfarb J. Slow freezing, vitrification and ultra-rapid freezing of human embryos: a systematic review and meta-analysis. Reproductive biomedicine online. 2010 Feb 1;20(2):209-22. https://doi.org/10.1016/j.rbmo.2009.11.013.

12. Huang MT, Lee RK, Lu CH, Chen YJ, Li SH, Hwu YM. The efficiency of conventional microscopic selection is comparable to the hyaluronic acid binding method in selecting spermatozoa for male infertility patients. Taiwanese Journal of Obstetrics and Gynecology. 2015 Feb 1;54(1):48-53. https://doi.org/10.1016/j.tjog.2014.11.006.

13. Huszar G, Jakab A, Sakkas D, Ozenci CC, Cayli S, Delpiano E, Ozkavukcu S. Fertility testing and ICSI sperm selection by hyaluronic acid binding: clinical and genetic aspects. Reproductive biomedicine online. 2007 Jan 1;14(5):650-63. https://doi.org/10.1016/S1472-6483(10)61060-7.

14. Irez T, Ocal P, Guralp O, Kaleli S, Ocer F, Sahmay S. Sperm selection based on motility in polyvinylpyrrolidone is associated with successful pregnancy and embryo development. Andrologia. 2013 Aug;45(4):240-7. https://doi.org/10.1111/j.1439-0272.2012.01337.x.

15. Endo Y, Fujii Y, Shintani K, Seo M, Motoyama H, Funahashi H. Simple vitrification for small numbers of human spermatozoa. Reproductive biomedicine online. 2012 Mar 1;24(3):301-7.https://doi.org/10.1016/j.rbmo.2011.11.016.

16. Kathrins M, Abhyankar N, Shoshany O, Liebermann J, Uhler M, Prins G, Niederberger C. Post-thaw recovery of rare or very low concentrations of cryopreserved human sperm. Fertility and Sterility. 2017 Jun 1;107(6):1300-4. https://doi.org/10.1016/j.fertnstert.2017.04.016.

17. Kato Y, Nagao Y. Effect of polyvinylpyrrolidone on sperm function and early embryonic development following intracytoplasmic sperm injection in human assisted reproduction. Reproductive medicine and biology. 2012 Oct;11(4):165-76.https://doi.org/10.1007/s12522-012-0126-9.

18. Amidi F, Pazhohan A, Shabani Nashtaei M, Khodarahmian M, Nekoonam S. The role of antioxidants in sperm freezing: a review. Cell and tissue banking. 2016 Dec;17(4):745-56. https://doi.org/10.1007/s10561-016-9566-5.

19. Liu Y, Feenan K, Chapple V, Roberts P, Matson P. Intracytoplasmic sperm injection using hyaluronic acid or polyvinylpyrrolidone: a time-lapse sibling oocyte study. Human Fertility. 2019 Jan 2;22(1):39-45. https://doi.org/10.1080/14647273.2017.1366077.

20. Montagut M, Gatimel N, Bourdet-Loubère S, Daudin M, Bujan L, Mieusset R, Isus F, Parinaud J, Leandri R. Sperm freezing to address the risk of azoospermia on the day of ICSI. Human Reproduction. 2015 Nov 1;30(11):2486-92.https://doi.org/10.1093/humrep/dev234.

21. Nabi A, Entezari F, Miresmaeili SM, Vahidi S, Lorian K, Anbari F, Motamedzadeh L. Evaluation of Sperm Parameters and DNA Integrity Following Different Incubation Times in PVP Medium. Urology journal. 2021 Oct 13:6936-.DOI: 10.22037/uj.v18i.6936. Epub ahead of print. PMID: 34655072.

22. Paasch U, Grunewald S, Agarwal A, Glandera HJ. Activation pattern of caspases in human spermatozoa. Fertility and sterility. 2004 Mar 1;81:802-9.https://doi.org/10.1016/j.fertnstert.2003.09.030.

23. Justice T, Christensen G. Sperm cryopreservation methods. InSpermatogenesis 2013 (pp. 209-215). Humana Press, Totowa, NJ.

24. Parmegiani L, Cognigni GE, Bernardi S, Troilo E, Taraborrelli S, Arnone A, Maccarini AM, Filicori M. Comparison of two ready-to-use systems designed for sperm–hyaluronic acid binding selection before intracytoplasmic sperm injection: PICSI vs. Sperm Slow: a prospective, randomized trial. Fertility and sterility. 2012 Sep 1;98(3):632-7.https://doi.org/10.1016/j.fertnstert.2012.05.043.

25. Koscinski I, Wittemer C, Lefebvre-Khalil V, Marcelli F, Defossez A, Rigot JM. Optimal management of extreme oligozoospermia by an appropriate cryopreservation programme. Human Reproduction. 2007 Oct 1;22(10):2679-84.https://doi.org/10.1093/humrep/dem190.