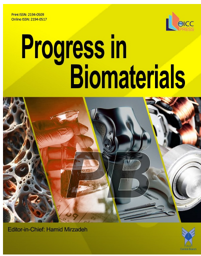

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Human Sperm Cryopreservation Using Polyvinyl Alcohol as a Cryoprotectant

Atefe Mohsennezhad[1,2], Maryam Ghobeh[1], Mohsen Sharafi[3], Saeed Zandiyeh[4], Marjan Sabbaghian[2,*]

[1] *Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran.*
[2] *Department of Andrology, Reproductive Biomedicine Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran.*
[3] *Department of Animal Science, College of Agriculture, Tarbiat Modares University.*
[4] *Department of Biology, Damghan Branch, Islamic Azad University of Damghan, Iran.*

[*] **Corresponding author:** m.sabbaghian@royaninstitute.org

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Original Research

Abstract:

Cryopreservation is a procedure commonly used worldwide to keep a broad range of living cells and tissues, such as sperm, oocytes, and embryos, at extremely low temperatures for long periods while retaining the integrity of their structures. In order to preserve fertility during cancer treatment, sperm cryopreservation has become a common practice. Polyvinyl alcohol (PVA), a synthetic polymer, has shown to prevent the growth of ice crystals and inhibit ice recrystallization at lower concentrations. In the current investigation, the aim was to assess the PVA as a supplement to improve human sperm cryopreservation. Sperm from 21 normospermic males were collected and divided into five equal groups: Fresh, frozen control, 0.01% PVA, 0.1% PVA, and 0.5% PVA. After being frozen and thawed, the main sperm parameters were examined, such as motility, viability, morphology, malondialdehyde (MDA) level, and DNA fragmentation. Based on the results, human sperm motility and viability were increased by adding 0.01% PVA in comparison with the control group. Moreover, different concentrations of PVA did not affect morphology, MDA, or DNA fragmentation. It is reasonable to declare that using an optimal concentration of impermeable ice inhibitors, such as PVA, in conjunction with a permeable cryoprotectant significantly improves the enhancement of human sperm after thawing.

Keywords:

Cryopreservation; Polyvinyl alcohol (PVA); Human sperm; Cryoprotectant

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1. Introduction

Cryopreservation is a method to maintain organelles, cells, and tissues at cryobiological temperatures for long periods (Di Santo et al., 2022; Jang et al., 2017). Infertility, which is attributed to changes in modern lifestyle, consumption of processed foods and products, exposure to hazardous chemicals, etc., is among the most important health issues in the world since it impacts not only the patients' social and psychological well-being, but also affects their sexual life (Daneshfar et al., 2023; Salas-Huetos et al., 2017; Bala et al., 2021; Durairajanayagam, 2018). Cryopreservation before infertility treatment can help future relevant patients to enjoy having babies (Sanger et al., 1992). Male fertility and assisted reproductive technology (ART), such as in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI), rely significantly on the process of sperm cryopreservation, which involves freezing sperm for future use (Li et al., 2019; Mohsennezhad et al., 2024). After the first pregnancy was successfully achieved by insemination using frozen-thawed sperm, human sperm cryopreservation was applied more than 50 years ago (Jeyendran et al., 2008). However, cryopreservation negatively impacts sperm parameters, including DNA integrity, motility, plasma membrane structure, and mitochondrial function (Castro et al., 2016; Gonzalez et al., 2022). The development of intracellular (IC) and extracellular (EC) ice crystals causes osmotic stress and thermal shock in the freeze-thaw procedures (Oldenhof et al., 2013). These conditions may change in cell volume, which might be fatal if it exceeds the osmotic tolerance limit. The pro-

duction of ice crystals in the IC follows ice nucleation in the EC space, which causes water to be extruded from the cell. However, in the case of rapid cooling of the cell, not sufficient water will be evacuated, resulting in the development of IC ice crystals and, eventually, cell death. Extreme shrinkage may occur if cells are exposed to high solute concentrations over extended periods, which can occur with even modest cooling rates (Zandiyeh et al., 2020). Furthermore, the cell was damaged by ice recrystallization during cryopreservation. Traditional cryoprotectants, including glycerol ($C_3H_8O_3$) and dimethylsulfoxide (DMSO), work through various processes; however, at the concentrations typically used in cryopreservation procedures, ice recrystallization is not inhibited or controlled by them (Briard et al., 2016). It is an interesting issue to find a way to preserve the quantity and quality of sperm in the freeze-thaw process; current methods and cryoprotective agents (CPAs) are not optimal regarding dilution rates, carrier instruments, CPAs composition, freezing rates, or thawing procedure (Li et al., 2019).

Polyvinyl Alcohol (PVA, $[CH_2CH(OH)]_n$) is the most robust polymer, similar to antifreeze glycoproteins, which prevents the growth of ice crystals at concentrations as low as 1 mg/mL. PVA is also used in an extensive range of medical and food applications and has been developed as a powerful cryoprotectant, which prevents ice growth and cell freezing with a complex relationship (Congdon et al., 2016). Cryoprotectants reduce the damage in the freezing process and are divided into two categories: (I) permeable and (II) impermeable to the cell membrane. The PVA belongs to the impermeable category and has the capacity to minimize the size of ice crystals (Jang et al., 2017). Consequently, PVA does not penetrate inside the cell, and it affects its outside environment (Deller et al., 2014). PVA establishes hydrogen bonds with the crystal molecules of ice due to the presence of OH groups. The PVA is attached to the Prism and Basal parts of ice crystals and thus prevents their growth (Budke and Koop, 2006). In order to prevent the freezing process, we should use controlled concentrations of less than 1 mg/mL (Mitchell et al., 2015). PVA co-polymers can be costly to produce in large quantities compared to antifreeze proteins (Wowk et al., 2000). Some studies indicated that using PVA as a new approach prevents ice crystal formation throughout the freezing (Mitchell et al., 2015).

The process of sperm freezing began with the freezing of bird sperm in 1957, and human sperm was first frozen in 1960 (Walters et al., 2009). In 2010, a research group used a copolymer of PVA and vinyl acetate named Supercool X-1000 to freeze mouse embryos and observed a significant increase in viability (Badrzadeh et al., 2010). Another group investigated the effect of PVA on freezing sheep sperm and found a significant increase in motility after a certain period (Corcini et al., 2010). In 2013, some studies were conducted on freezing human and sheep RBCs using PVA, in which the cell recovery was increased (Deller et al., 2014). In 2019, a group of researchers studied the application of a combination of PVA as a non-permeable cryoprotectant and glycerol as a permeable cryoprotectant in the post-thaw process via the significant mitochondrial activity, acrosome integrity, viability, and motility in frozen goat sperm. The use of a low percentage of PVA is feasible and effectively protects vital physiological parameters of sperm (Tekin and Daşkın, 2019). Another research published in 2019 suggests that adding PVA to Tris extenders without egg yolk (EY) protects dog sperm after freezing without affecting acrosome motility or integrity. In addition, sperm motility may be recovered after being frozen by lowering the pH of PVA supplements without egg yolk to 6.85. Hence, PVA may be used as an additive in cryoprotective agents without egg yolk to freeze dog sperm (Nabeel et al., 2019).

The present study investigated the impacts of various concentrations of 9 kDa and 30 kDa PVA as a cryoprotectant similar to antifreeze proteins in human sperm cryopreservation. Ultimately, we investigated the optimal concentration of PVA in the freezing medium containing glycerol-egg yolk-citrate (GEYC) after the process of sperm cryopreservation. The motility, viability, lipid peroxidation, DNA fragmentation, and morphology were evaluated.

2. Materials and methods

2.1 Semen collection and analysis

An experimental interventional investigation was conducted. Twenty-one normospermic males provided their semen samples by masturbation after $3-7$ days of sexual abstinence. Leukocytospermia ($\geq 1 \times 10^6$ cells/mL), varicocele, endocrine abnormalities, medicine usage, antioxidant use, smoking cigarettes, alcohol use, and chemotherapy or radiation exposure were all exclusion criteria. A basic semen analysis was performed in accordance with World Health Organization (WHO) guidelines after the samples were liquefied at 37 °C for 40 min. All samples must have met the following standards for acceptance: normal viscosity; sperm count $> 35 \times 10^6$ sperm/mL; sperm motility $> 40\%$. Then, the dilution process of semen samples was performed using a GEYC-based freezing medium. For the experimental groups, three concentrations of 0.01%, 0.1%, and 0.5% of PVA co-polymers were added to the freezing medium. The used PVA derivatives (Sigma-Aldrich) included 9 kDa PVA (average Mw $9000-10000$, 80% hydrolyzed) and 30 kDa PVA ($30,000-70,000$, $98-99\%$ hydrolyzed). First, three concentrations of PVA co-polymers (9 kDa and 30 kDa) were tested. By observing a significant increase in the sperm motility percentage in PVA (9 kDa) with a concentration of 0.01%, the study was continued using the 0.01% PVA (9 kDa). Subsequently, one of the three groups, including fresh, frozen control (untreated sperm), and frozen groups, was assigned to each sample exposed to 0.01% PVA before cryopreservation.

2.2 Cryopreservation and thawing procedure

The sperm samples were frozen in the GEYC medium, then placed into cryo-straws, and frozen by exposure to a vapor of liquid nitrogen (LN2) for 15 min at a distance of 5 cm from the LN2 surface. Afterward, the cryo-straws were submerged in LN2 and stored for one week. The cryo-straws containing the frozen sperm were thawed in a water bath at 37 °C for 30 s. The cryoprotectants were eliminated through washing the samples with Ham's F-10

medium supplemented with 10% HSA and centrifuging them at 3000 rpm for 5 min. Afterward, the sperm pellets obtained at the end were re-suspended in 100 μL of Ham's F-10 medium (Zandiyeh et al., 2020; Beirão et al., 2012; Qadeer et al., 2014; Nishijima et al., 2014).

2.3 Sperm motility

The protocol for evaluating sperm motion characteristics was the same as the protocol presented in a previous research by Zandiyeh et al. (2020) and Alipour et al. (2017). The protocol is presented in Section S.1 of the Supplementary Information. It is noteworthy that the sperm progression was categorized as progressive motile, non-progressive motile, and immotile.

2.4 Sperm viability

Eosin-nigrosin (EoNig) solution (for preparation protocol, refer to (Zandiyeh et al., 2020) or Section S.2 in the Supplementary Information was used to assess sperm viability. On a glass slide, equal volumes of EN and sperm were smeared together. Light microscopy (Olympus CX21, Tokyo, Japan) was applied to analyze the dried slides, and each sample was evaluated using 200 sperm. By applying this method, the non-viable sperm were stained as dark pink or deep red, while the viable sperm looked white and light pink (Muiño et al., 2008).

2.5 Morphology

A glass slide was employed to make the sperm smears, fixed in methyl alcohol, and then stained with Papanicolaou staining after being air-dried at room temperature. Per-slide evaluations of sperm cells were made by strict criteria (Cooper et al., 2010). Under a phase-contrast microscope (Olympus CKX41, Tokyo, Japan), the 200 spermatozoa were selected to determine the percentage of normal morphology according to a previous study (Hezavehei et al., 2019).

2.6 DNA fragmentation

Sperm DNA fragmentation was assessed using the method presented in previous research conducted by (Hosseinmardi et al., 2022). This method is described in detail in Section S.3 of the Supplementary Information. It should be noted that the DNA was classified as fragmented in sperm cells with small or nonexistent halos or that had been degraded, and as intact in those with larger or medium-sized halos.

2.7 Lipid peroxidation

Seminal plasma was assessed for malondialdehyde (MDA) concentrations. The seminal plasma and sperm pellet were obtained after the samples were centrifuged at 1500 rpm for 5 min to assess the MDA content. After a 30-minute incubation at 95 °C, seminal plasma was chilled for 5 min on ice to measure malondialdehyde concentration. The absorbance of the supernatant was determined by chemiluminescence at 530 – 540 nm after a 5-minute centrifugation of the samples at 1500 rpm.

3. Results

3.1 Post-thaw evaluation of sperm

3.1.1 Motion characteristics

Spermatozoa's total motility, progressive motility, and viability were studied to see how they were affected by PVA concentrations of varying molecular weights. There was no significant difference in total sperm motility between the control (0.00%), 0.01%, and 0.5% of PVA (9 kDa and 30 kDa) groups; however, there was a significant ($P < 0.05$) increase in the percentage of sperm motility in the 0.01% PVA (9 kDa) group (figure 1).

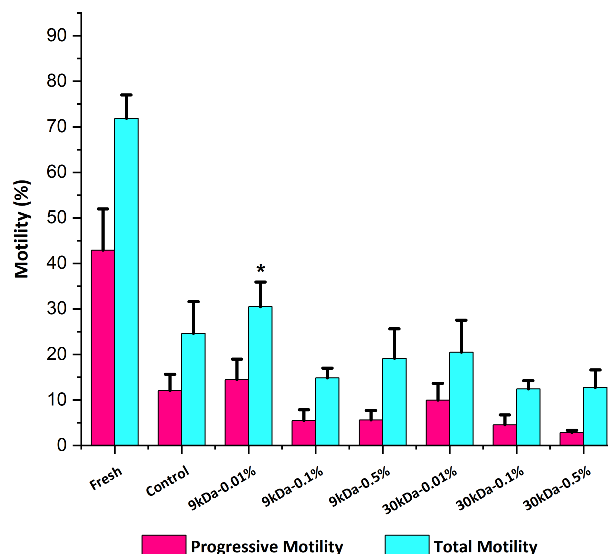

Figure 1. Effect of different concentrations of PVA on the percentage of motility of frozen-thawed human sperm. The data are presented as mean ± SD (n = 6). Sperm total motility and progressive motility in the Fresh Control group were significantly higher than those in Frozen Control and Frozen 0.01% PVA (9 kDa) groups ($P < 0.05$); *Sperm Total motility in the Frozen 0.01% PVA (9 kDa) was significantly higher compared to that in Frozen Control ($P < 0.05$).

The 0.01% concentration of PVA with the most effective was chosen for further investigations. Table 1 and figure 2 showed the mean ± SE and mean ± SD values, in respective order, for the measured sperm parameters after thawing sperm cryopreserved in either fresh control, frozen control (GEYC medium), or frozen PVA. The fresh control had greater total motility and progressive parameters than those of both the frozen control and frozen PVA ($P < 0.05$). Compared with the frozen control, frozen PVA shows significant overall motility ($P < 0.05$). In comparison to the frozen control, the percentage of immotile sperm was also smaller in the fresh control and frozen PVA ($P < 0.05$). Moreover, frozen PVA had a significantly ($P < 0.05$) lower value of this parameter than in the fresh control.

3.1.2 Viability

Percentages of viable sperm in each experimental group are shown in figure 3. Accordingly, the percentage of live cells in frozen PVA was statistically higher ($P < 0.05$) than in the frozen control group. As compared to the fresh group, both frozen groups demonstrated significantly lower values ($P < 0.05$).

Table 1. Effects of PVA in GEYC medium on human sperm motility. The data are presented as mean ± SE; n = 21.

Motility (%)	Fresh	Frozen control	Frozen PVA 0.01% (9 kDa)
Total motility	75.41 ± 1.57	29.26 ± 2.14	37.02 ± 2.67*
Progressive motile	50.99 ± 3.30	14.10 ± 1.46	17.46 ± 1.71
Non-progressive motile	23.12 ± 1.30	15.31 ± 1.17	18.79 ± 6.03
Immotile	24.67 ± 1.75	67.49 ± 3.16	63.28 ± 2.62

*P < 0.05 compared with the frozen control group.

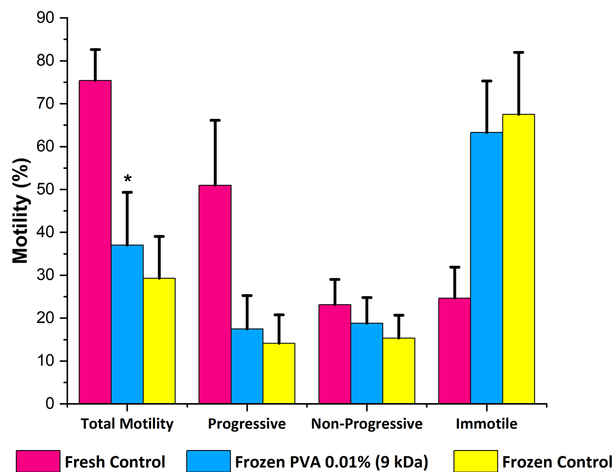

Figure 2. Effects of 0.01% PVA (9 kDa) in GEYC medium on human sperm motility. Data are expressed as mean ± SD; n = 21. Sperm motion characteristics in the Fresh Control group were significantly higher than in Frozen Control and Frozen 0.01% PVA (9 kDa) groups ($P < 0.05$); *Sperm Total motility in the Frozen 0.01% PVA (9 kDa) was significantly higher compared to that in the Frozen Control ($P < 0.05$).

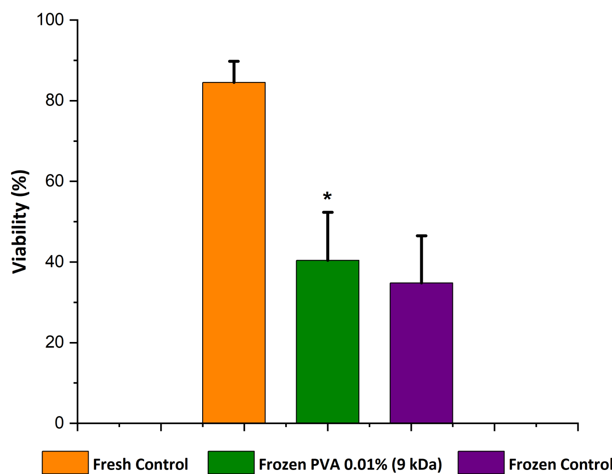

Figure 3. Effects of 0.01% PVA (9 kDa) in GEYC medium on human sperm viability. Data are expressed as mean ± SD; n=21. Sperm viability in the Fresh Control group was significantly higher than that in the Frozen Control and Frozen 0.01% PVA (9 kDa) groups ($P < 0.05$); *Sperm viability in the Frozen 0.01% PVA (9 kDa) was significantly higher compared to that in the Frozen Control ($P < 0.05$).

3.1.3 Morphology and DNA fragmentation

The effects of PVA on morphology and DNA fragmentation were evaluated and presented in figure 4. Fresh control sperm had a higher normal morphology than frozen control

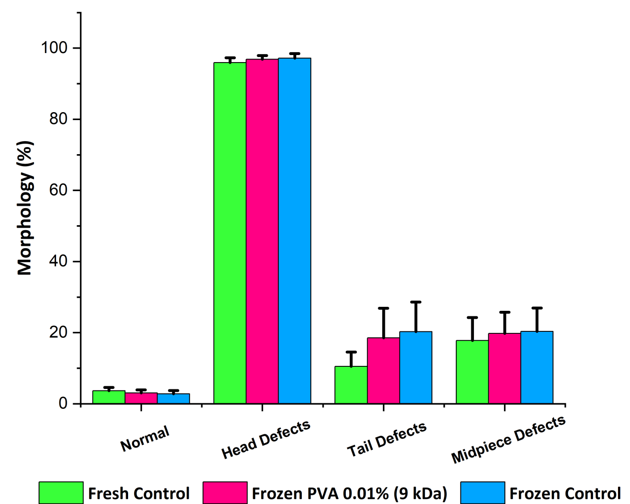

Figure 4. Effects of 0.01% PVA (9 kDa) in GEYC medium on human sperm morphology. Data are expressed as mean ± SD; n = 21. The number of normal sperms in the Fresh Control group was significantly higher than that in the Frozen Control and Frozen 0.01% PVA (9 kDa) groups. Head and tail defects morphology in the Frozen Control and Frozen PVA were significantly higher compared to that in the Fresh Control ($P < 0.05$).

as well as frozen PVA ($P < 0.05$). Morphological defects in the head and tail were evident in the frozen control and frozen PVA compared to the fresh control. The frozen PVA and the control group showed no significant difference. The DNA fragmentation index was much lower in fresh than in frozen control and frozen PVA ($P < 0.05$; figure 5). Moreover, there was no significant difference in this value between the frozen control and the frozen PVA.

3.1.4 Lipid peroxidation

The MDA levels in seminal plasma were significantly ($P < 0.05$) lower in frozen PVA and frozen control than in fresh control. Regarding the results obtained from the frozen control and frozen PVA samples, there was no statistically significant difference ($P < 0.05$; figure 6).

4. Discussion and conclusion

Cryopreservation is a common method for keeping cells and tissues alive at very low temperatures. However, cryodamage to the cells may result from freezing and thawing, which can reduce viability and motility, alter morphology, break DNA, and promote lipid peroxidation. Cryoprotectants and ice inhibitors are two strategies that have been investigated to lessen the severity of these

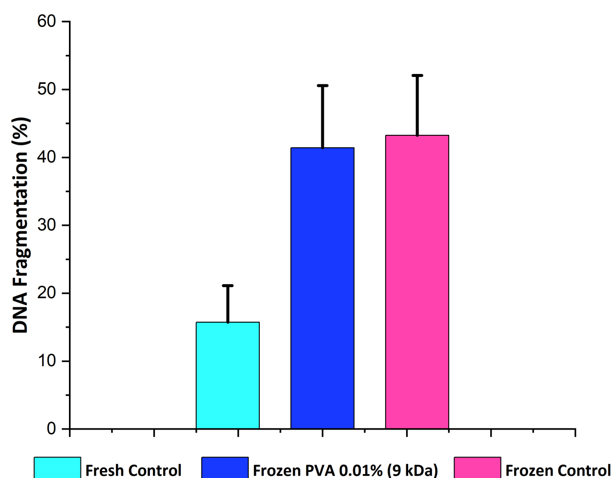

Figure 5. Effects of 0.01% PVA (9 kDa) in GEYC medium on human sperm DNA fragmentation. Data are expressed as mean ± SD; n = 21. DNA fragmentation in the Frozen Control and Frozen 0.01% PVA (9 kDa) group was significantly higher compared to that in the Fresh Control ($P < 0.05$).

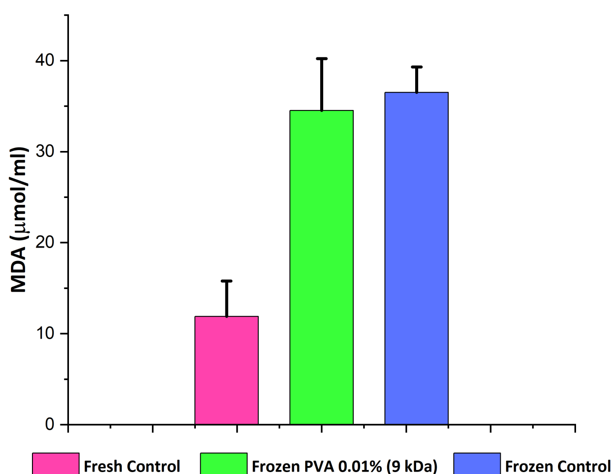

Figure 6. Effects of 0.01% PVA (9 kDa) in GEYC medium on human sperm MDA level. Data are expressed as mean ± SD; n = 21. The MDA level in the Frozen Control and Frozen 0.01% PVA (9 kDa) group was significantly higher compared to that in the Fresh Control ($P < 0.05$).

side effects and boost cryopreservation success (Jaiswal et al., 2022; Lusignan et al., 2018). This research looked at whether the synthetic polymer PVA might be used to enhance the cryopreservation of human sperm. The findings showed that sperm motility was dramatically enhanced when 0.01% PVA was added to the mixture. This result agrees with prior research showing that PVA may prevent the recrystallization of ice. PVA, which is unable to penetrate the sperm membrane, protects the sperm's exterior structure against the development of ice crystals (Fayter et al., 2020; Bachtiger et al., 2021; Bleszynski, 2022; Naullage and Molinero, 2020; Sabando et al., 2022). When compared to the frozen control group, the samples that had been treated with PVA before being frozen exhibited greater overall motility and progressive measurements after being thawed. Furthermore, in the frozen PVA group, the proportion of immotile sperm was considerably lower than in the frozen control group. These results imply that adding PVA to frozen sperm helps them move more freely and decreases the number of immobile sperm. Additionally, the frozen PVA group had considerably greater sperm viability than the frozen control group. PVA helped keep more sperm alive after freezing and thawing, even though both frozen groups had lower viability than the fresh control group. Notably, the morphology of sperm, the rate at which DNA was fragmented, or the rate at which lipids were oxidized were not affected by the presence of PVA. When comparing the morphological flaws and DNA fragmentation of the frozen PVA and control groups, there were no discernible changes. Furthermore, both the frozen PVA and control groups had decreased levels of malondialdehyde (MDA), a sign of lipid peroxidation, compared to the fresh control group. These results demonstrate that PVA supplementation has no effect on the morphology, DNA, or lipid peroxidation of sperm.

In conclusion, PVA may be used as a supplement to enhance human sperm cryopreservation. After freezing, the motility and viability of sperm were greatly improved by the addition of PVA at a concentration of 0.01%. Sperm morphology, DNA fragmentation, and lipid peroxidation were all unaffected by PVA supplementation. These results suggest that PVA has promise as a cryoprotectant and ice inhibitor for protecting sperm integrity and viability during transportation over long distances. To improve the concentration and use of PVA in various cryopreservation methods, further research is required. It is also important to study PVA's long-term impact on fertilization rates and embryo growth. Improved cryopreservation procedures for reproductive reasons will benefit from a deeper understanding of the processes behind the cryoprotective properties of PVA and its interactions with other cryoprotectants.

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Ethical Statement
It was clear to all participants how the study would be conducted and how their clinical and biological data would be used. All subjects provided written informed consent and permitted the scientific community to use their sperms for medical investigations, and the Royan Institute's Ethics Committee approved the research procedure (EC: IR.ACECR.ROYAN.REC.1397.185).

Authors contributions

All authors contributed equally to the conception, design, execution, and writing of this work. All authors read and approved the final manuscript.

Availability of data and materials
The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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