



Technological and clinical advances in sperm cryopreservation: A literature review

Postępy technologiczne i kliniczne w kriokonserwacji nasienia – przegląd piśmiennictwa

Jakub Nocoń¹ , Artur Spyрка¹ , Szymon Domagała¹ , Kamil Kania¹ , Michał Stojko¹ ,
Mateusz Marcinek² , Michał Tkocz² 

¹Students' Scientific Club, Department of Urology, Faculty of Medical Sciences in Katowice,
Medical University of Silesia, Katowice, Poland

²Department of Urology, Faculty of Medical Sciences in Katowice, Medical University of Silesia, Katowice, Poland

ABSTRACT

Cryopreservation of sperm is a mandatory fertility preservation technique, particularly for preventing fertility loss among oncological patients. As technology advances toward better freezing protocols to maximize post-thaw sperm quality, this review presents current applications, efficacy, and progress in the field, focusing on new cryoprotectants, vitrification protocols, and quality-facilitating strategies. The analysis covers innovations such as vitrification with cryoprotectant microinjection, cryoprotectant-free strategies, and the addition of natural antioxidants (γ -tocopherol or *Sargassum* extract) or pharmacological agents (pentoxifylline, melatonin, or caffeine). The review also addresses clinical outcomes in special populations, including oncological patients, men with Klinefelter syndrome, HIV-1-positive individuals, and patients with disorders of sex development. Although clinical evidence demonstrates that cryopreservation significantly enhances the potential of preserving fertility, the utilization rates of stored specimens remain low. Currently, a distinguishable transition is observed from conventional slow-freezing protocols to vitrification strategies and modifications of the cryogenic environment that reduce cellular injury. However, due to small sample sizes and differences in protocols among studies, it has been noted that there exists a need to standardize practices, identify predictive biomarkers, and adapt protocols for an ever-changing population of patients. Further research in multicenter randomized controlled trials would be important to identify effective ways of optimizing this method.

KEYWORDS

antioxidants, male infertility, sperm cryopreservation, vitrification, cryoprotectants, fertility preservation

Received: 23.12.2025

Revised: 06.01.2025

Accepted: 18.01.2026

Published online: 20.03.2026

Address for correspondence: Kamil Kania, Studenckie Koło Naukowe, Oddział Kliniczny Urologii, Wojewódzki Szpital Specjalistyczny Nr 5 im. św. Barbary, pl. Medyków 1, 41-200 Sosnowiec, tel. +48 32 368 25 11, e-mail: kania.kamil.lek@gmail.com



This is an open access article made available under the terms of the Creative Commons Attribution-ShareAlike 4.0 International (CC BY-SA 4.0) license, which defines the rules for its use. It is allowed to copy, alter, distribute and present the work for any purpose, even commercially, provided that appropriate credit is given to the author and that the user indicates whether the publication has been modified, and when processing or creating based on the work, you must share your work under the same license as the original. The full terms of this license are available at <https://creativecommons.org/licenses/by-sa/4.0/legalcode>.

Publisher: Medical University of Silesia, Katowice, Poland



STRESZCZENIE

Kriokonserwacja nasienia odgrywa kluczową rolę w zabezpieczeniu płodności, nabierając krytycznego znaczenia u pacjentów onkologicznych, u których leczenie systemowe grozi utratą funkcji rozrodczych. W obliczu postępu technologicznego ukierunkowanego na optymalizację protokołów mrożenia niniejszy przegląd analizuje aktualne zastosowania oraz skuteczność nowych rozwiązań w tej dziedzinie. Szczególną uwagę poświęcono innowacyjnym krioprotektantom, protokołom witrifikacji oraz strategiom służącym poprawie jakości nasienia po rozmrożeniu. W pracy omówiono różne podejścia, w tym techniki mikroiniekcyjne, protokoły wolne od klasycznych krioprotektantów, wspomaganie naturalne (γ -tokoferol czy ekstrakt z *Sargassum*) oraz farmakologiczne (pentoksyfilina, melatonina i kofeina). Zakres analizy poszerzono o specyficzne grupy kliniczne: pacjentów onkologicznych, pacjentów z zespołem Klinefeltera, zakażonych wirusem HIV-1 oraz pacjentów z zaburzeniami rozwoju płci. Co istotne, wysoka skuteczność laboratoryjna procedur nie koreluje z częstością wykorzystania próbek, gdyż pacjenci rzadko decydują się na ich użycie. Obserwuje się wyraźną tendencję do odchodzenia od konwencjonalnego, powolnego mrożenia na rzecz witrifikacji, która skuteczniej minimalizuje uszkodzenia strukturalne. Niemniej jednak, z uwagi na ograniczoną liczebność grup badawczych oraz heterogeniczność stosowanych protokołów kluczowe staje się ujednoczenie praktyk laboratoryjnych. Niezbędna jest identyfikacja biomarkerów predykcyjnych oraz dostosowanie metod mrożenia do ewoluujących potrzeb zróżnicowanej populacji pacjentów. Warunkiem dalszego postępu jest realizacja szeroko zakrojonych, wielośrodkowych badań, które zweryfikują użyteczność kliniczną dostępnych rozwiązań.

SŁOWA KLUCZOWE

antyoksydanty, niepłodność męska, kriokonserwacja nasienia, witrifikacja, krioprotektanty, zachowanie płodności

Introduction

Sperm cryopreservation has a long history dating back to the late 18th century, when Lazzaro Spallanzani first described the effect of low temperatures on sperm motility [1]. A breakthrough discovery by Christopher Polge, Audrey Smith, and Alan Parkes in 1949, identifying glycerol as an effective cryoprotectant [2], initiated the development of clinical sperm freezing, which since the 1960s has become the cornerstone of fertility preservation in assisted reproductive techniques (such as in vitro fertilization [IVF] or intracytoplasmic sperm injection [ICSI]), primarily for oncological patients. With technological advances leading to novel methods of sperm cryopreservation, this technique has become increasingly valuable as both a therapy for male infertility and a means of preserving fertility in at-risk individuals.

Patients with cancers such as lymphoma, leukemia, or testicular cancer typically undergo vigorous treatment interventions, i.e., chemotherapy, radiotherapy, or radical surgery, which can cause irreversible sterility. Thus, sperm cryopreservation before the onset of such treatment protocols is seriously advised by various scientific organizations, including the American Society of Clinical Oncology (ASCO). The literature testifies that 8%–9% of oncological patients eventually make use of their cryopreserved spermatozoa, a percentage predicted to increase with time [3]. Likewise, cryopreservation is also utilized for men with chronic and progressive oligozoospermia

or non-obstructive azoospermia, where complete fertility loss becomes higher with increasing duration. It is also commonly done preoperatively for urological surgery whose side effects jeopardize testicular function and surgeries for retrieving sperm, such as testicular sperm extraction (TESE) [4].

This narrative review synthesizes papers retrieved from the PubMed database up to April 2025, focusing on English-language clinical trials and randomized controlled trials published primarily from 2010 onwards. We used keywords related to “sperm cryopreservation” and “male fertility preservation,” excluding animal models to prioritize recent technological and clinical advancements in human reproduction. The selected studies are deemed pivotal for illustrating the current applications and latest technological advancements in semen cryopreservation, with a particular emphasis on freezing methods, novel cryoprotectants, and strategies for optimizing post-thaw semen quality.

Key studies

To provide a concise overview of the most relevant studies discussed in this work, Table I summarizes key publications on sperm cryopreservation, including methodological modifications and clinical applications. It presents their authorship, year of publication, study population, applied technique, and main findings, thereby facilitating a quick comparison and identification of research trends in this field.



Table I. Overview of key studies on sperm cryopreservation, methodological modifications, and clinical applications

Author	Study population (n)	Study type	Cryopreservation technique / Intervention	Key outcomes
1	2	3	4	5
Han et al. [14]	10 patients with normal spermatogenesis, testicular tissue samples	In vitro study	Vitrification with cryoprotectant microinjection (VLP) vs. vitrification without microinjection vs. slow freezing	VLP significantly reduced spermatogonial apoptosis ($p = 0.047$), improved seminiferous tubule integrity, and enhanced post-thaw sperm motility
Slabbert et al. [15]	35 semen samples from healthy HIV–negative men	In vitro study	Conventional cryopreservation (TEST-yolk buffer) vs. vitrification without penetrating cryoprotectants	Vitrification improved mitochondrial potential and reduced DNA fragmentation; no difference in motility
Zerbinati et al. [25]	134 semen samples	In vitro study	Cryopreservation with α - and γ -tocopherol supplementation	γ -tocopherol enhanced sperm viability and motility post-thaw; strong antioxidant activity protected sperm during cryopreservation
Sobhani et al. [27]	11 healthy men	In vitro study	Rapid cryopreservation with <i>Sargassum</i> extract (250/500 mg/mL)	Extract significantly reduced ROS and improved motility; no effect on sperm morphology
Xian et al. [30]	68 azoospermic patients	In vitro study	Cryopreservation with pentoxifylline supplementation	Pentoxifylline significantly improved sperm motility before and after cryopreservation; no effect on viability
Pariz et al. [33]	30 normozoospermic men	In vitro study	Slow freezing with glycerol buffer; melatonin before and caffeine after thawing	Melatonin and caffeine improved motility and mitochondrial activity; reduced oxidative stress
Fortunato et al. [39]	103 subfertile men	Prospective cohort study	Slow freezing with seminal plasma preserved	Cryopreservation increased chromatin decondensation and DNA damage depending on morphology and storage duration
Frainais et al. [40]	20 healthy men and 20 HIV-1–positive men	Prospective cohort study	Sperm selection with density gradient; cryopreservation with glycerol	HIV-1–positive men had higher baseline DNA fragmentation; gradient selection reduced fragmentation; freezing/thawing did not worsen DNA damage but reduced motility and viability
Záková et al. [41]	523 men with testicular cancer (283 seminoma, 240 NSGCT)	Retrospective cohort study	Standard slow freezing in liquid nitrogen	6.5% used frozen sperm; 46 ART cycles with frozen sperm achieved pregnancy in 34.8% of cases (ICSI: 34.2%; IUI: 50%)
van der Kaaij et al. [42]	902 men treated for Hodgkin's lymphoma; 363 banked sperm	Retrospective cohort study	Pre-treatment sperm cryopreservation, standard slow freezing	40% cryopreserved sperm; 21% of them used samples; cryopreservation doubled fatherhood rates after treatment (OR = 2.03)



1	2	3	4	5
González-Ravina et al. [43]	181 donor insemination cycles; high-quality donors	Randomized controlled trial	MACS after DGC vs. DGC alone before insemination	MACS reduced sperm concentration but improved progressive motility; no significant differences in pregnancy, live birth, or miscarriage rates
Brugnon et al. [44]	64 men with oligoasthenoteratozoospermia	In vitro study	Cryopreservation with sperm selection by density gradient, with or without hypotaurine	Pre-freeze selection improved motile sperm recovery and mitochondrial potential; hypotaurine reduced oxidative stress and apoptosis markers; selection before freezing lowered DNA fragmentation
Plotton et al. [45]	41 men with azoospermia and non-mosaic 47,XXY karyotype	Prospective cohort study	Bilateral open TESE; cryopreservation if ≥ 20 sperm were retrieved	Overall SRR = 56.1%; no age effect; prior testosterone therapy not significant; successful pregnancies and live births achieved via ICSI with thawed sperm
Johnson et al. [46]	10 children with DSD undergoing gonadectomy	Retrospective case series	Gonadal tissue cryopreservation during prophylactic gonadectomy	Germ cells present in 50% of cases, more frequent in younger patients; 4/5 families with germ cells opted for freezing; fertility potential uncertain, but procedure feasible and promising

VLP – vitrification with low CPA (cryoprotective agents) concentration and microinjection; HIV – human immunodeficiency virus; TEST – TES-Tris; DNA – deoxyribonucleic acid; ROS – reactive oxygen species; NSGCT – non-seminomatous germ cell tumors; ART – assisted reproductive technology; ICSI – intracytoplasmic sperm injection; IUI – intrauterine insemination; MACS – magnetic-activated cell sorting; DGC – density gradient centrifugation; TESE – testicular sperm extraction; SRR – sperm retrieval rate; DSD – disorders of sex development

Comparison of sperm cryopreservation methods

Sperm cryopreservation remains a fertility preservation standard of care prior to oncological treatment for cancer patients. The majority of antineoplastic therapy – including chemotherapy, radiotherapy, and surgery – produces irreversible impacts to the genital tract. For this reason, fertility preservation should be integrated into treatment planning as part of survivorship care [5]. Today, the two most influential strategies utilize cryopreservation: slow freezing and vitrification. The main differences between the two lie in their respective cryoprotectant concentrations and cooling rates, which directly affect possibilities for ice crystal development and subsequent structural and functional damages to spermatozoa [6]. Slow freezing functions by slowly replacing intracellular water with cryoprotectants to minimize cellular harm, with cooling rates being adjusted according to sperm membrane permeability properties [7]. Advantages to utilizing this method include a lower threat of pathogenic infection, moderate to low amounts of cryoprotectant dosage, and low skill level demands by operators. However, one considerable setback is a possible extracellular development of ice crystal structures that can harm sperm cells [8]. Vitrification, however, encompasses a very quick transition of sperm suspension from

a liquid to a glassy state after treatment with liquid nitrogen [9]. Such a transition necessitates pre-exposure to higher grades of cryoprotectants (usually 40%–60%, w/v), together with quick cooling to prevent nucleation of ice [10,11]. The chief benefit of vitrification is its low predisposition to freeze cell injury. There is a list of specific setbacks to such a technique, however, including a higher risk of contamination with pathogenic microbes as well as the requirement for advanced technical expertise [8].

Recent developments in safer sperm cryopreservation

The above-mentioned strategies are restricted by the variable penetration of cryoprotectants through tissues, which could lead to different degrees of protection and add to the threat of cell disruption throughout the freezing and thawing procedures. It is a serious problem mostly within parts of seminiferous tubules, since close tissue texture inhibits passive penetration of the cryoprotectants [12,13]. To overcome such a challenge, Han et al. [14] explored a method of microinjection of vitrification solution directly into seminiferous tubules before vitrification. Testicular tissues were retrieved from 10 normospermic individuals and stratified into four groups: fresh control, standard slow freezing with 1.5 M and 2.1 M dimethyl sulfoxide



(DMSO), vitrification with or without microinjection, and vitrification with microinjection, referred to as vitrification with low CPA concentration and microinjection (VLP). Vitrification solution microinjection was carried out with micropipettes to allow accurate delivery of the cryoprotectants into the tubule lumen to increase distribution efficiency and minimize osmotic gradients between intra- and extracellular compartments. The results showed that VLP usage significantly reduced the proportion of apoptotic spermatogonia compared with both standard vitrification ($p = 0.047$) and conventional freezing protocols ($p = 0.049$). Moreover, VLP showed better preservation of seminiferous tubule integrity and supported better recovery of spermatozoa with progressive motility after thawing, particularly when standard 0.25 ml cryogenic straws were used.

Due to the high toxicity of the penetrating cryoprotective agents (CPAs) needed for the standard vitrification method, Slabbert et al. [15] developed a novel CPA-free vitrification method. In comparisons with standard slow freezing (TES-Tris [TEST]-yolk buffer), it utilized a solution with sucrose (0.25 M) and with human serum albumin (1%) as osmotic substitutes. One operational benefit of such a method was having the ability to vitrify high volumes of samples (up to 300 μ l), thereby increasing its practical value for clinical applications. Post-thaw analysis showed that sperm motility parameters, i.e., progressive motility and total motility, were similar between CPA-free vitrification and slow freezing ($p > 0.05$). However, significantly higher mitochondrial membrane potential was noted after CPA-free vitrification ($11.99\% \pm 4.33\%$) than after slow freezing ($6.58\% \pm 1.03\%$, $p < 0.001$), indicating higher energy storage and metabolism after thawing. Moreover, the percentage of spermatozoa with deoxyribonucleic acid (DNA) fragmentation was significantly lower after CPA-free vitrification ($2.79\% \pm 1.02\%$) compared to standard cryopreservation ($3.86\% \pm 1.38\%$, $p < 0.01$).

Innovative strategies for sperm protection

While fertility preservation is the general goal of cryoprotection, the process itself is one of the leading causes of sperm damage. Despite a number of protective attributes – i.e., low cytoplasmic volume [16] and an unusual lipid membrane configuration (high cholesterol content and a low ratio of unsaturated to saturated fatty acids) [17] – human spermatozoa are strongly susceptible to membrane disruption, motility dysfunction, and excessive reactive oxygen species (ROS) production, with the resultant oxidative stress and threat to DNA integrity. To counteract these effects, specific protective agents come into play. These protectants are called cryoprotectants, which represent the core premise behind freezing protocols of semen. Two major categories emerge from the current literature:

- Penetration cryoprotectants (e.g., DMSO, glycerol, or ethylene glycol), which permeate through the

plasma membrane, create intracellular osmotic gradients to prevent ice crystal growth and to stabilize cell membranes

- Non-penetrating cryoprotectants (e.g., sucrose, trehalose, or egg yolk lipoproteins), which are extracellular and allow water efflux, thus maintaining plasma membrane integrity upon cooling.

The most commonly employed formulations contain glycerol as the principal permeating entity, with sugars and egg yolk serving as non-permeating carriers [18] and most often supplemented with antibiotics to regulate microbial presence.

Despite their critical role, cryoprotectants have several limitations. Vitrification involves a high concentration of these compounds, which markedly increases their toxicity [8]. Moreover, cases have been described where sperm with apparently normative motility and morphometry after thawing still contain hidden injury – this is an especially serious problem with ICSI, where a single sperm is directly placed into the oocyte [19]. In addition, cryoprotectants can also potentially disrupt mitochondrial membrane integrity with the resultant excessive release of ROS yielding oxidative stress. These events can culminate in oxidative DNA lesions, such as 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is an established marker for oxidative DNA damage [20,21].

Antioxidants as a new defense mechanism

Oxidative stress has been described as an important contributor to sperm injury [22], largely owing to the high amounts of polyunsaturated fatty acids present in sperm membranes. Lipid peroxidation has adverse effects on membrane permeability, structural integrity, and membrane fluidity, which reduce motility as well as fertilizing ability [23]. Under normal physiological conditions, semen contains several enzymatic antioxidants, including superoxide dismutase and catalase, as well as low-molecular-weight antioxidants such as ascorbic acid and α -tocopherol, one of the four isomeric forms of vitamin E [24].

In a study by Zerbinati et al. [25], adding γ -tocopherol to the cryoprotection medium significantly improved sperm survival and motility after thawing compared to α -tocopherol or controls. Although both isomers had antioxidant effects, γ -tocopherol selectively scavenged nitric oxide and thus possibly played a key role in the protection of sperm membranes against oxidative insult during freezing. Note that the authors emphasized the importance of mixing tocopherols because α -tocopherol alone could replace γ -tocopherol on the membranes, thereby increasing susceptibility to oxidative stress.

The findings on vitamin E isomers highlight the need to explore alternative antioxidants that may enhance sperm protection during cryopreservation. In recent years, special attention has been paid to natural compounds, particularly polyphenol-rich plant extracts. A notable innovation is the use of *Sargassum*



(brown algae) extract, which exhibits strong in vitro antioxidant activity due to its high content of phenolic compounds – especially flavonoids [26]. In a study by Sobhani et al. [27], *Sargassum* extract significantly reduced ROS production ($p < 0.005$) and, at concentrations of 250 and 500 mg/ml, markedly increased total motility ($p < 0.006$) and progressive motility ($p < 0.007$) after freezing, without affecting sperm morphology.

Pharmacological additives supporting cryopreservation

The limitations of standard cryoprotectants justify continuing to seek new compounds that can enhance semen conservation. Various pharmacological agents acting on energy metabolism and mitochondrial integrity have been explored in recent years. One example is pentoxifylline (PF), a phosphodiesterase inhibitor commonly utilized to stimulate epididymal and testicular sperm motility [28]. PF exerts its effect by inhibiting cyclic adenosine monophosphate (cAMP) phosphodiesterase, resulting in higher intracellular levels of cAMP. Cellular respiration is stimulated, motility is improved, and acrosome reaction is induced [29]. In 2021, Xian et al. [30] compared a PF-supplemented cryoprotectant with a non-PF-supplemented cryoprotectant in 68 azoospermic patients, divided into a control group (cryoprotectant only) and an experimental group (cryoprotectant with PF 2.5 mmol/l). Following thawing, motile sperm were present in 100% of the experimental samples, compared to 44.1% of the controls. Motility recovery after thawing was significantly higher among the experimental group than in the controls (70.1% vs. 38.2%, $p = 0.038$), with no difference between groups regarding sperm viability ($p = 0.237$).

Melatonin has also been explored as a cryopreservation adjunct. Melatonin is a potent antioxidant that is excellent for mitochondrial function as well as stimulating ATP production by boosting respiratory chain oxidative phosphorylation complex I and IV [31]. Caffeine, by contrast, inhibits phosphodiesterase activity to enhance intracellular cAMP levels and is also a protein kinase A activator, boosting cytochrome c oxidase activity and oxidative phosphorylation [32,33]. Pariz et al. [33] divided semen from 30 healthy normospermic men (19–45 years old) into four groups: melatonin supplementation (2 mM) prior to freezing, caffeine (2 mM) supplementation after thawing, a combination treatment (melatonin prior to freezing and caffeine after thawing), and an untreated standard TEST-yolk buffer control. Total and progressive motility were determined after thaw analysis supplemented with mitochondrial activity, DNA fragmentation index, ROS concentration, ROS levels, and viability. The caffeine and combination groups showed considerable improvement in progressive motility ($p = 0.005$ and $p = 0.048$, respectively). Mitochondrial activity significantly improved with the

combination treatment ($p = 0.001$). DNA fragmentation did not significantly vary across groups; ROS levels did not significantly differ across groups either.

Impact of cryopreservation on sperm chromatin and DNA integrity

Semen cryopreservation is a standard fertility preservation approach among men who receive gonadotoxic therapy such as radical surgery, radiotherapy, or chemotherapy [34,35]. It can be stored for long periods with fertility preservation. Nonetheless, several studies have confirmed that freezing together with long-term storage is linked with DNA lesions as well as disruption of chromatin, with a direct impact concerning the effectiveness of assisted reproductive treatment and an adverse health threat among offspring [36,37,38].

Damage to chromatin and DNA fragmentation following cryopreservation

In 2012, Fortunato et al. [39] conducted a study on 103 men evaluated for reduced fertility, aiming to assess the impact of cryopreservation and storage duration (10 and 90 days) on sperm chromatin condensation using aniline blue staining. The results showed that the mean sperm decondensation index (SDI) increased by 156% after 10 days and by 331% after 90 days of liquid nitrogen storage ($p < 0.05$). Impaired chromatin integrity was particularly evident in patients with abnormal baseline semen parameters. At baseline, SDI was significantly higher in oligospermic men ($21.9 \pm 3.4\%$) compared to normozoospermic men ($14.0 \pm 0.9\%$, $p < 0.01$). Likewise, men with severe teratozoospermia (<4% normal forms) exhibited higher SDI ($19.6 \pm 1.8\%$) than those with moderate morphological defects (4%–14% normal forms [$12.6 \pm 1.1\%$]). At day 0, however, no significant correlation was observed between abnormal SDI and other semen parameters such as progressive motility or pH alterations [39].

The other related aspect is regarding the effect of cryopreservation on DNA integrity among human immunodeficiency virus type 1 (HIV-1)-infected individuals. Infections and treatment impacts on frozen semen quality were measured by researchers. There were 40 men enrolled in the study and then divided into two groups. There were 20 healthy HIV-negative men who were used as controls (mean age: 38.07 ± 5.93 years), while 20 HIV-1-infected men (mean age: 41.44 ± 5.80 years) who had been receiving antiretroviral therapy for >6 months with an undetected viral load and had a CD4+ T cell count of $>200/\text{mm}^3$ (mean: $645 \pm 72/\text{mm}^3$) were part of an investigative group. Sperm DNA fragmentation was determined on three occasions: fresh semen, after density-gradient selection, and after freezing.

Baseline DNA fragmentation was significantly higher in the HIV-1 group compared to the controls (6.38% vs. 3.39%, $p < 0.05$). Selection against sperm with DNA



fragmentation markedly decreased sperm with DNA fragmentation in both groups (to 2.62% for HIV-1 patients and 1.50% for controls, $p < 0.05$). Note that after cryopreservation, a significant rise compared with baseline fresh semen results did not occur, with results being 5.18% for the HIV group and 3.40% for the controls ($p = \text{NS}$). In the HIV-1 men, no correlations were found with duration of antiretroviral therapy, CD4+ T cell count, or consumption of alcohol or tobacco [40].

Clinical applications and effectiveness of sperm freezing in selected patients

Preservation of fertility prior to gonadotoxic treatment

A retrospective analysis by Záková et al. [41] assessed the long-term results of sperm cryopreservation with subsequent usage in men with testicular cancer receiving oncological treatment. The retrospective analysis spanned 18 years (October 1995–December 2012) and comprised 557 men diagnosed with testicular cancer, among whom sperm was successfully cryopreserved in 523 (mean age: 28.5 years). Seminoma represented the most frequent histological subtype (54.1%), followed by non-seminomatous germ cell tumors (NSGCT; 45.9%). Thirty-four men (6.1%) were diagnosed with azoospermia that precluded cryopreservation; they were thus excluded from further analysis. Pre-freeze semen analysis showed normospermia in only 5.6% of men, with the most frequent aberrations being oligoasthenoteratozoospermia (OAT; 53.1%), then oligo-/asthenozoospermia (35.2%). At long-term follow-up, only 34 men (6.5%) had used their cryopreserved samples for infertility treatment. A total of 46 assisted reproductive technology (ART) cycles were undertaken: six intrauterine inseminations (IUI), with three pregnancies (50%) and one live birth, and 38 ICSI cycles with 13 pregnancies (34.2%) and five live births. The total pregnancy rate/treatment cycle was 34.8%. The mean time to utilize samples after cryopreservation was 22.2 ± 14.7 months.

In a large cohort study, van der Kaaij et al. [42] assessed sperm cryopreservation outcomes among 902 men treated for Hodgkin lymphoma between 1974 and 2004. Of these, only 363 patients (40%) opted for sperm banking prior to gonadotoxic therapy. Factors influencing this decision included younger age (≤ 29 years), higher education (aOR: 1.60, 95% CI: 1.11–2.30, $p = 0.01$), advanced disease stage (III/IV) reducing the likelihood of cryopreservation (aOR: 0.54, 95% CI: 0.32–0.92, $p = 0.02$), and chemotherapy treatment (aOR: 4.26, 95% CI: 2.55–7.13, $p < 0.001$) or second-line therapy (aOR: 2.03, 95% CI: 1.18–3.49, $p = 0.01$). Among the 363 patients who cryopreserved sperm, only 78 ultimately used their samples for ART. Of all men who became fathers after Hodgkin lymphoma treatment ($n = 258$), 19% (48 cases)

achieved fatherhood with cryopreserved sperm. Importantly, having banked sperm doubled the likelihood of post-treatment fatherhood (adjusted OR: 2.03, 95% CI: 1.11–3.73, $p = 0.02$).

Additional methods for enhancing semen quality after thawing

In a prospective, multicenter randomized trial, González-Ravina et al. [43] examined whether magnetic-activated cell sorting (MACS) after thawing would enhance the results of IUI with sperm from donors. A total of 181 cycles were randomly divided into two groups: standard density gradient centrifugation (DGC [controls]) and DGC with subsequent MACS (MACS group). Semen parameters after thawing showed substantial differences in sperm concentration: 36.76 million/mL (95% CI: 32.34–41.19) in the controls versus 23.44 million/mL (95% CI: 19.84–27.04) in the MACS group, with a mean difference of 13.3 million/mL ($p < 0.001$). Progressive motility was significantly higher in the experimental group (86.69%, 95% CI: 84.94–88.44) compared to the controls (82.51%, 95% CI: 80.95–84.07) with a mean difference of 4.18 percentage points ($p < 0.001$). Neither semen volume nor sperm morphology were significantly different. Despite these improved parameters, clinical outcomes were comparable between the groups. Clinical pregnancy rates per cycle were 26.7% (MACS group) and 26.4% (controls) (RR: 1.01, 95% CI: 0.623–1.642, $p = 0.96$). Live birth rates per cycle were also higher in the MACS group (58.3% vs. 50%), but without statistical significance (RR: 1.17, 95% CI: 0.69–1.97, $p = 0.56$). Miscarriage rates were slightly lower in the MACS group (41.7% vs. 50%), but again not statistically significant (RR: 0.83, 95% CI: 1.45–1.55, $p = 0.56$).

In 2013, Brugnon et al. [44] reported on the effects of sperm selection from hypotaurine supplementation combined with DGC prior to cryopreservation on semen quality in men with OAT. This prospective, randomized study analyzed semen from 64 men with OAT, divided into four groups (16 samples each): (1) pre-freeze DGC without hypotaurine, (2) pre-freeze DGC with hypotaurine, (3) post-thaw DGC without hypotaurine, and (4) post-thaw DGC with hypotaurine. The findings demonstrated that pre-freeze DGC significantly improved post-thaw sperm quality compared to post-thaw DGC. Improvements were observed across multiple markers, including progressive motility (recovery rate: $2.6\% \pm 0.84$ vs. $1.4\% \pm 0.31$, $p = 0.04$), mitochondrial potential ($58.1\% \pm 3.50$ vs. $46.7\% \pm 5.48$, $p = 0.001$), reduced DNA fragmentation ($38.6\% \pm 9.59$ vs. $55.7\% \pm 5.88$, $p = 0.001$), and lower phosphatidylserine expression ($25.0\% \pm 2.10$ vs. $20.2\% \pm 3.63$, $p = 0.01$). Hypotaurine significantly decreased the proportion of viable sperm with phosphatidylserine expression when used in the pre-freeze selection setting (6.8% vs. 11.8%, $p = 0.04$). In other conditions, hypotaurine supplementation had no significant effect.



Special patient populations requiring a specialized approach

Some patient groups presenting for sperm cryopreservation necessitate special strategies because they present with distinctive clinical and pathophysiological characteristics. One such category is men with Klinefelter syndrome. Plotton et al. [45] assessed TESE efficacy for non-mosaic Klinefelter patients. They recruited 41 azoospermic men with a confirmed karyotype of 47,XXY and divided them into a younger cohort (15–23 years, $n = 25$) and an adult cohort (23 years or older, $n = 16$). The sperm retrieval rate (SRR) overall was 56.1% (23/41). Successful retrieval occurred in 52% of the younger age group and 62.5% in the older group with no statistical difference ($p = 0.73$). Testosterone replacement therapy prior to treatment did not adversely affect retrieval results (SRR: 52.9% with vs. 59.1% without therapy, $p = 0.98$). Hormonal markers and seminiferous tubular volume were non-prognostic with respect to TESE. All except one of these men were azoospermic. Sperm were successfully retrieved from 23 of them. Seven couples went on to ICSI. Ten cycles were attempted (three cycles for one couple, two cycles for one couple, and one cycle each for five couples), with four pregnancies being established and three live births resulting; one further pregnancy was ongoing at the time of publication.

Another population that benefits from advancements in cryopreservation technique is patients with disorders of sex development (DSD). Johnson et al. [46] examined 10 children with various forms of DSD (mixed gonadal dysgenesis, androgen insensitivity syndrome, and Y chromosome material bearing Turner syndrome) undergoing gonadectomy and counseled regarding gonadal tissue cryopreservation (GTC). Their mean age was 11.5 years (range: 1–18) and nine of them were assigned female sex at birth. Histological examination diagnosed germ cells in five patients' gonadal tissues, four of whom proceeded with GTC. Eight patients underwent bilateral gonadectomy, whereas two underwent unilateral surgery. Three cases demonstrated gonadoblastoma, one of which displayed dysgerminoma. Pre-gonadectomy multidisciplinary meetings were conducted with patients and their parents. Interestingly enough, the presence and quality of germ cells were associated with younger age of the patient.

Discussion

The current literature recognizes a clear shift away from conventional slow-freezing techniques, which have been followed for decades, toward new technologies such as vitrification and novel approaches involving direct microinjection of cryoprotectants into seminiferous tubules [14,15]. Interestingly, Han et al. [14] reported that direct injection of cryoprotectants into seminiferous tubules overcomes the barrier of dense tissue texture that typically inhibits passive

cryoprotectant permeation. Consequently, this method greatly reduced spermatogonial apoptosis and improved post-thaw motility of sperm, thereby improving the outcome of vitrification ($p = 0.047$). However, this study was limited by its small sample size ($n = 10$) and one must be skeptical of the external validity of its findings.

Another notable contribution is from Slabbert et al. [15], who managed to achieve vitrification using non-penetrating cryoprotectants in similarly high sample volumes (300 μ l). Their results indicated reduced DNA fragmentation and increased mitochondrial potential of spermatozoa, which is an improvement compared to the original studies in the 2000s that utilized microscale volumes of less than 20 μ l [47].

Antioxidants play an important role in inhibiting cryoinjury. Such substances as γ -tocopherol [25] and flavonoids of *Sargassum* spp. [27] were found to dramatically increase the viability and motility of thawed sperm and to reduce ROS levels. Such findings highlight the potential of natural antioxidants as effective substitutes for traditional pharmacological compounds. However, differences in chemical stability, bioavailability, and modes of action between γ -tocopherol and phlorotannins underscore the need for further investigations. In particular, maximum dosing, delivery strategies, clinical efficacy during cryopreservation conditions, and the long-term effects on ART are still under-investigated. Pharmacological agents such as PF [30] and melatonin + caffeine [33] have also been found to be helpful, particularly in improving motility and mitochondrial function, but their action on DNA integrity appears limited. However, a brief comment is warranted on whether such pharmacological stimulation might merely improve motility while masking underlying genetic damage. This potential “masking effect” is highly interesting to the ART community, as it raises the risk of selecting DNA-fragmented sperm for ICSI solely based on recovered motility.

Despite the evolution of technology, long-term sperm storage remains problematic due to higher DNA fragmentation and alterations in chromatin condensation, effects which are notably relevant for abnormal baseline semen parameters or comorbid patients [39,40]. These molecular alterations are crucial from a clinical standpoint, most notably in the context of ART procedures such as ICSI. Fortunato et al. [39] reported a sharp increase in chromatin decondensation after 90 days of storage, with a more pronounced impact in oligozoospermic men. Similarly, Frainais et al. [40] demonstrated that HIV-1-positive subjects did have higher baseline DNA fragmentation, which was not further exacerbated after cryopreservation, thus establishing the safety of such interventions in this population when using a standard sperm selection protocol.

From a clinical perspective, cohort and retrospective studies confirm the central role of sperm cryopreservation as a method of preserving fertility for



oncology patients. Záková et al. [41] reported that just 6.5% of men with testicular cancer indeed used their frozen sperm for ART, and the resulting pregnancy rate was 34.8%. Conversely, van der Kaaij et al. [42] reported that exposure to frozen sperm doubled the likelihood of fatherhood after treatment for Hodgkin lymphoma (OR: 2.03, $p = 0.02$).

Certain patient groups also gain significantly from sperm banking. For example, Plotton et al. [45] had an SRR of 56.1% ($n = 41$) in men with Klinefelter syndrome, with no correlation with age; more importantly, seven couples conceived using ICSI. In children with DSD, the use of GTC is increasingly known as an effective fertility-sparing technique. Johnson et al. [46] demonstrated that germ cells in 50% of pediatric patients undergoing gonadectomy can be stored, with increased retrieval rates among younger patients. This not only emphasizes the viability of experimental cryopreservation in this rare population, but also stresses the importance of early intervention in preserving future reproductive capabilities.

Adjunct therapies to improve post-thaw sperm quality, including MACS and DGC with hypotaurine supplementation, have been found to confer positive biological impacts. González-Ravina et al. [43] also recorded a rise in progressive motility by 4.18% following MACS, albeit with non-significant improvement in pregnancy. Brugnon et al. [44] also demonstrated that DGC with hypotaurine supplementation improves mitochondrial potential, decreases DNA fragmentation, and lowers apoptosis. Though biologically important, the long-term clinical consequences of such techniques are not known.

The limitations of the studies in this review largely relate to the small sample numbers, which restrict the statistical power of their findings. For example, the landmark study by Han et al. [14] on direct microinjection of cryoprotectants used just 10 samples, while the trial by Sobhani et al. [27] of *Sargassum* extract used only 11 specimens. Additionally, heterogeneity is extreme between cryopreservation protocols and sperm testing methodologies. Differences in the composition and concentration of the selected cryoprotectants (e.g., glycerol, sucrose, or DMSO), freezing protocol (slow freezing vs. vitrification), and thaw conditions complicate the comparability of the results. Similarly, heterogeneity of the results – e.g., post-thaw motility, DNA fragmentation, mitochondrial function, and ROS levels – complicates the process of developing standardized clinical recommendations [39,40,41].

Another important limitation is the lack of well-validated and established predictive biomarkers that can assess the success of cryopreservation and forecast its long-term impact on sperm function. Of particular concern in this context is genetic damage, including increased DNA fragmentation and compromised

chromatin condensation, which may significantly affect ART success rates [39,40].

Subsequent research must involve large, multicenter randomized clinical trials to compare the efficacy and safety of new approaches such as direct cryoprotectant microinjection [14] and vitrification with non-permeating cryoprotectants [15]. Additional research into antioxidant [25,27] and pharmacological [30,33] supplements as potential post-thaw sperm quality promoters is warranted. Last but not least, standardized cryopreservation protocols and semen assessment methods are required, particularly in the populations that require a specially adapted clinical protocol: men with Klinefelter syndrome [45], children with DSD [46], and oncological patients [41,42]. Harmonizing diagnostic criteria and individualizing protocols can potentially optimize the effectiveness and safety of sperm cryopreservation and therefore optimize therapeutic outcomes and pave the way for future recommendations.

Conclusions

Sperm cryopreservation technology seems to be moving away from traditional slow freezing procedures toward more innovative techniques such as vitrification. New approaches – e.g., direct cryoprotectant microinjection or cryoprotectant-free techniques – are emerging. In addition to these breakthroughs in technology, recent trends show that additives in the cryopreservation environment hold promise. Natural antioxidants such as γ -tocopherol or *Sargassum* extract have demonstrated protective functions against oxidative stress. Pharmacological agents – e.g., PF or melatonin-caffeine combinations – have been observed to improve crucial parameters such as motility.

Applications in clinical settings are highly relevant to patients with malignancies and other selected groups. Notably, this review reveals efforts to achieve retrieval and subsequent cryopreservation in complex patients with Klinefelter syndrome or children with DSD. Nevertheless, in spite of these encouraging observations, the available data has been limited by small patient numbers and heterogeneous protocols. Thus, randomized clinical trials are warranted to establish evidence-based protocols to ensure safety and efficacy in fertility preservation. In fact, future efforts should aim to establish reliable predictive biological markers and promote the global harmonization of diagnostic criteria. Such standardization is essential in order to devise individualized protocols to ensure safety and efficacy for fertility preservation.

Conflict of interest

The authors declare no conflicts of interest.

**Authors' contribution**

Study design – J. Nocoń, M. Stojko, S. Domagała, M. Tkocz
Data collection – A. Spyryka, J. Nocoń, K. Kania, M. Marcinek
Manuscript preparation – J. Nocoń, K. Kania, M. Stojko, A. Spyryka, S. Domagała
Literature research – S. Domagała, A. Spyryka, K. Kania, M. Stojko
Final approval of the version to be published – M. Tkocz, M. Marcinek

REFERENCES

1. Doetsch RN, Lazzaro Spallanzani's Opuscoli of 1776. *Bacteriol Rev.* 1976;40(2):270–275. doi: 10.1128/br.40.2.270-275.1976.
2. Szein JM, Takeo T, Nakagata N. History of cryobiology, with special emphasis in evolution of mouse sperm cryopreservation. *Cryobiology.* 2018;82:57–63. doi: 10.1016/j.cryobiol.2018.04.008.
3. Ferrari S, Paffoni A, Filippi F, Busnelli A, Vegetti W, Somigliana E. Sperm cryopreservation and reproductive outcome in male cancer patients: a systematic review. *Reprod Biomed Online.* 2016;33(1):29–38. doi: 10.1016/j.rbmo.2016.04.002.
4. Esteves SC. Clinical management of infertile men with nonobstructive azoospermia. *Asian J Androl.* 2015;17(3):459–470. doi: 10.4103/1008-682X.148719.
5. Su HI, Lacchetti C, Letourneau J, Partridge AH, Qamar R, Quinn GP, et al. Fertility Preservation in People With Cancer: ASCO Guideline Update. *J Clin Oncol.* 2025;43(12):1488–1515. doi: 10.1200/JCO.2024.02782.
6. Gao D, Critser JK. Mechanisms of Cryoinjury in Living Cells. *ILAR J.* 2000;41(4):187–196. doi: 10.1093/ilar.41.4.187.
7. Yong KW, Wan Safwani WK, Xu F, Wan Abas WA, Choi JR, Pingguan-Murphy B. Cryopreservation of Human Mesenchymal Stem Cells for Clinical Applications: Current Methods and Challenges. *Biopreserv Biobank.* 2015;13(4):231–239. doi: 10.1089/bio.2014.0104.
8. Jang TH, Park SC, Yang JH, Kim JY, Seok JH, Park US, et al. Cryopreservation and its clinical applications. *Integr Med Res.* 2017;6(1):12–18. doi: 10.1016/j.imr.2016.12.001.
9. Rall WF, Fahy GM. Ice-free cryopreservation of mouse embryos at –196°C by vitrification. *Nature.* 1985;313(6003):573–575. doi: 10.1038/313573a0.
10. Yavin S, Arav A. Measurement of essential physical properties of vitrification solutions. *Theriogenology.* 2007;67(1):81–89. doi: 10.1016/j.theriogenology.2006.09.029.
11. Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online.* 2005;11(3):300–308. doi: 10.1016/s1472-6483(10)60837-1.
12. Cordeiro RM, Stirling S, Fahy GM, De Magalhães JP. Insights on cryoprotectant toxicity from gene expression profiling of endothelial cells exposed to ethylene glycol. *Cryobiology.* 2015;71(3):405–412. doi: 10.1016/j.cryobiol.2015.10.142.
13. Curaba M, Verleysen M, Amorim CA, Dolmans MM, Van Langendonck A, Hovatta O, et al. Cryopreservation of prepubertal mouse testicular tissue by vitrification. *Fertil Steril.* 2011;95(4):1229–1234.e1. doi: 10.1016/j.fertnstert.2010.04.062.
14. Han S, Zhao L, Yang C, Xu J, Yao C, Huang C, et al. Vitrification with microinjection of single seminiferous tubules: an efficient cryopreservation approach for limited testicular tissue. *Reprod Biomed Online.* 2021;43(4):687–699. doi: 10.1016/j.rbmo.2021.06.026.
15. Slabbert M, du Plessis SS, Huysen C. Large volume cryoprotectant-free vitrification: an alternative to conventional cryopreservation for human spermatozoa. *Andrologia.* 2015;47(5):594–599. doi: 10.1111/and.12307.
16. Isachenko E, Isachenko V, Katkov II, Dessole S, Nawroth F. Vitrification of mammalian spermatozoa in the absence of cryoprotectants: from past practical difficulties to present success. *Reprod Biomed Online.* 2003;6(2):191–200. doi: 10.1016/s1472-6483(10)61710-5.
17. Bailey JL, Bilodeau JF, Cormier N. Semen Cryopreservation in Domestic Animals: A Damaging and Capacitating Phenomenon. *J Androl.* 2000;21(1):1–7. doi: 10.1002/j.1939-4640.2000.tb03268.x.
18. Hezavehei M, Sharafi M, Kouchesfahani HM, Henkel R, Agarwal A, Esmaili V, et al. Sperm cryopreservation: A review on current molecular cryobiology and advanced approaches. *Reprod Biomed Online.* 2018;37(3):327–339. doi: 10.1016/j.rbmo.2018.05.012.
19. Tamburrino L, Traini G, Marcellini A, Vignozzi L, Baldi E, Marchiani S. Cryopreservation of Human Spermatozoa: Functional, Molecular and Clinical Aspects. *Int J Mol Sci.* 2023;24(5):4656. doi: 10.3390/ijms24054656.
20. Gualtieri R, Kalthur G, Barbato V, Longobardi S, Di Rella F, Adiga SK, et al. Sperm Oxidative Stress during In Vitro Manipulation and Its Effects on Sperm Function and Embryo Development. *Antioxidants.* 2021;10(7):1025. doi: 10.3390/antiox10071025.
21. Thomson LK, Fleming SD, Aitken RJ, De Iulius GN, Zieschang J-A, Clark AM. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. *Hum Reprod.* 2009;24(9):2061–2070. doi: 10.1093/humrep/dep214.
22. Ko EY, Sabanegh ES Jr, Agarwal A. Male infertility testing: reactive oxygen species and antioxidant capacity. *Fertil Steril.* 2014;102(6):1518–1527. doi: 10.1016/j.fertnstert.2014.10.020.
23. Potts RJ, Notarianni LJ, Jefferies TM. Seminal plasma reduces exogenous oxidative damage to human sperm, determined by the measurement of DNA strand breaks and lipid peroxidation. *Mutat Res.* 2000;447(2):249–256. doi: 10.1016/s0027-5107(99)00215-8.
24. Kefer JC, Agarwal A, Sabanegh E. Role of antioxidants in the treatment of male infertility. *Int J Urol.* 2009;16(5):449–457. doi: 10.1111/j.1442-2042.2009.02280.x.
25. Zerbinati C, Caponecchia L, Fiori C, Sebastianelli A, Salacone P, Ciacciarelli M, et al. Alpha- and gamma-tocopherol levels in human semen and their potential functional implications. *Andrologia.* 2020;52(4):e13543. doi: 10.1111/and.13543.
26. Duan XJ, Zhang WW, Li XM, Wang BG. Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata*. *Food Chem.* 2006;95(1):37–43. doi: 10.1016/j.foodchem.2004.12.015.
27. Sobhani A, Eftekhari TE, Shahrzad ME, Natami M, Fallahi S. Antioxidant Effects of Brown Algae Sargassum on Sperm Parameters: CONSORT-Compliant Article. *Medicine.* 2015;94(52):e1938. doi: 10.1097/MD.0000000000001938.
28. Angelopoulos T, Adler A, Krey L, Licciardi F, Noyes N, McCullough A. Enhancement or initiation of testicular sperm motility by in vitro culture of testicular tissue. *Fertil Steril.* 1999;71(2):240–243. doi: 10.1016/s0015-0282(98)00434-8.
29. Tournaye H, Janssens R, Verheyen G, Devroey P, Van Steirteghem A. In vitro fertilization in couples with previous fertilization failure using sperm incubated with pentoxifylline and 2-deoxyadenosine. *Fertil Steril.* 1994;62(3):574–579. doi: 10.1016/s0015-0282(16)56948-9.
30. Xian Y, Jiang M, Liu B, Zhao W, Zhou B, Liu X, et al. A cryoprotectant supplemented with pentoxifylline can improve the effect of freezing on the motility of human testicular sperm. *Zygote.* 2022;30(1):92–97. doi: 10.1017/S0967199421000368.
31. Succu S, Berlinguer F, Pasciu V, Satta V, Leoni GG, Naitana S. Melatonin protects ram spermatozoa from cryopreservation injuries in a dose-dependent manner. *J Pineal Res.* 2011;50(3):310–318. doi: 10.1111/j.1600-079X.2010.00843.x.
32. Pariz JR, Hallak J. Effects of caffeine supplementation in post-thaw human semen over different incubation periods. *Andrologia.* 2016;48(9):961–966. doi: 10.1111/and.12538.
33. Pariz JR, Ranéa C, Monteiro RAC, Evenson DP, Drevet JR, Hallak J. Melatonin and Caffeine Supplementation Used, Respectively, as Protective and Stimulating Agents in the Cryopreservation of Human Sperm Improves Survival, Viability, and Motility after Thawing compared to Traditional TEST-Yolk Buffer. *Oxid Med Cell Longev.* 2019;2019:6472945. doi: 10.1155/2019/6472945.
34. Gandini L, Lombardo F, Lenzi A, Spanò M, Dondero F. Cryopreservation and Sperm DNA Integrity. *Cell Tissue Bank.* 2006;7(2):91–98. doi: 10.1007/s10561-005-0275-8.
35. Trottmann M, Becker AJ, Stadler T, Straub J, Soljanik I, Schlenker B, et al. Semen Quality in Men with Malignant Diseases before and after Therapy and the Role of Cryopreservation. *Eur Urol.* 2007;52(2):355–367. doi: 10.1016/j.eururo.2007.03.085.
36. Watson PF. The causes of reduced fertility with cryopreserved semen. *Anim Reprod Sci.* 2000;60–61:481–492. doi: 10.1016/s0378-4320(00)00099-3.
37. Schuffner A, Morshedi M, Oehninger S. Cryopreservation of fractionated, highly motile human spermatozoa: effect on membrane phosphatidylserine externalization and lipid peroxidation. *Hum Reprod.* 2001;16(10):2148–2153. doi: 10.1093/humrep/16.10.2148.
38. Zribi N, Feki Chakroun N, El Euch H, Gargouri J, Bahloul A, Ammar Keskes L. Effects of cryopreservation on human sperm deoxyribonucleic acid integrity. *Fertil Steril.* 2010;93(1):159–166. doi: 10.1016/j.fertnstert.2008.09.038.
39. Fortunato A, Leo R, Liguori F. Effects of cryostorage on human sperm chromatin integrity. *Zygote.* 2013;21(4):330–336. doi: 10.1017/S0967199421000032.
40. Frainais C, Vialard F, Rougier N, Aegerther P, Damond F, Ayel JP, et al. Impact of freezing/thawing technique on sperm DNA integrity in HIV-1 patients. *J Assist Reprod Genet.* 2010;27(7):415–421. doi: 10.1007/s10815-010-9417-4.
41. Záková J, Lousová E, Ventruba P, Crha I, Pochopová H, Vinklárková J, et al. Sperm Cryopreservation before Testicular Cancer Treatment and Its Subsequent Utilization for the Treatment of Infertility. *ScientificWorldJournal.* 2014;2014:575978. doi: 10.1155/2014/575978.



42. van Der Kaaij MA, van Echten-Arends J, Heutte N, Meijnders P, Abeillard-Lemoisson E, Spina M, et al. Cryopreservation, semen use and the likelihood of fatherhood in male Hodgkin lymphoma survivors: an EORTC-GELA Lymphoma Group cohort study. *Hum Reprod.* 2014;29(3):525–533. doi: 10.1093/humrep/det430.
43. González-Ravina C, Santamaría-López E, Pacheco A, Ramos J, Carranza F, Murria L, et al. Effect of Sperm Selection by Magnetic-Activated Cell Sorting in D-IUI: A Randomized Control Trial. *Cells.* 2022;11(11):1794. doi: 10.3390/cells11111794.
44. Brugnon F, Ouchchane L, Pons-Rejraji H, Artonne C, Farigoule M, Janny L. Density gradient centrifugation prior to cryopreservation and hypotaurine supplementation improve post-thaw quality of sperm from infertile men with oligoasthenoteratozoospermia. *Hum Reprod.* 2013;28(8):2045–2057. doi: 10.1093/humrep/det253.
45. Plotton I, d'Estaing SG, Cuzin B, Brosse A, Benchaib M, Lornage J, et al. Preliminary Results of a Prospective Study of Testicular Sperm Extraction in Young Versus Adult Patients With Nonmosaic 47,XXY Klinefelter Syndrome. *J Clin Endocrinol Metab.* 2015;100(3):961–967. doi: 10.1210/jc.2014-3083.
46. Johnson EK, Finlayson C, Finney EL, Harris CJ, Tan SY, Laronda MM, et al. Gonadal Tissue Cryopreservation for Children with Differences of Sex Development. *Horm Res Paediatr.* 2019;92(2):84–91. doi: 10.1159/000502644.
47. Isachenko V, Isachenko E, Montag M, Zaeva V, Krivokharchenko I, Nawroth F, et al. Clean technique for cryoprotectant-free vitrification of human spermatozoa. *Reprod Biomed Online.* 2005;10(3):350–354. doi: 10.1016/s1472-6483(10)61795-6.