Sperm cryopreservation for impaired spermatogenesis

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Lay summary
Cryopreservation involves freezing of cells or tissues to preserve them for future use. Sperm cryopreservation for men with a very low sperm count is one of the commonest reasons for short-term sperm storage, usually in advance of fertility treatment. Cryopreservation is generally very effective, although not all sperm cells survive the process of freezing and thawing. This review covers various aspects of freezing sperm, including consideration of methods used and mechanisms of cell damage.
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Abstract
Sperm cryopreservation for men with severely impaired spermatogenesis is one of the commonest reasons for short-term sperm storage, usually in advance of fertility treatment. Cryopreservation is generally very effective, although not all spermatozoa survive the process of freezing and thawing. This review considers various aspects of freezing sperm, including an overview of methods, appropriate use of cryoprotectants and practical considerations, as well as oxidative stress and mechanisms of cell cryodamage.

Lay summary
Cryopreservation involves freezing of cells or tissues to preserve them for future use. Sperm cryopreservation for men with a very low sperm count is one of the commonest reasons for short-term sperm storage, usually in advance of fertility treatment. Cryopreservation is generally very effective, although not all sperm cells survive the process of freezing and thawing. This review covers various aspects of freezing sperm, including consideration of methods used and mechanisms of cell damage.
Introduction

Cryopreserved spermatozoa are used routinely in assisted reproductive technology (ART) worldwide. The origin of sperm cryobiology spans back several centuries to 1776 when Lazzarro Spallanzani, an Italian priest and scientist, first exposed and observed human spermatozoa (known as animalcules at the time) to different temperatures. His discovery that “heat kills the animalcules” was of key importance to subsequent discovery of sterilisation by Louis Pasteur. Crucially, he also reported that animalcules could survive and resume swimming after being exposed to snow and near-freezing temperatures. At the time, however, the fundamental basics of biology (sperm, egg, fertilisation) had yet to be fully understood. Almost a century later, Paolo Mantegazza published observations regarding survival of human spermatozoa after exposure to below-freezing conditions (Mantegazza, 1866). Ever the visionary, he concluded “If the human sperm can remain unchanged for more than four days at the melting ice temperature, it is certain that the science of the future will improve the breeds of horses and oxen, without forcing the enormous expense of transporting stallions and bulls, and they will be able to make artificial fertilization with the frozen sperm, shipped at great speed from one country to another. It may also be that a husband dead on the battlefields can fertilize his wife from his cadaver and have legitimate children even after he died.”

Historical scientific advances facilitated and contributed to the evolution of cryopreservation, including the use of liquid nitrogen to freeze and preserve sperm (Hoagland and Pincus, 1942) as well as studies by Parkes (Parkes, 1945) that demonstrated that slow freezing was less harmful to spermatozoa than rapid cooling, concluding that human sperm should be frozen in larger volume ampoules or tubes. But a key discovery was the use of glycerol as a cryoprotectant (Polge et al., 1949). As with many scientific revelations, the cryoprotective property of glycerol was discovered by chance when a series of experiments demonstrated unexpectedly high survival and motility of fowl spermatozoa after exposure to −79°C. Addition of fructose solution to spermatozoa was usual practice at the time, to achieve partial dehydration prior to freezing. A bottle kept in the laboratory fridge containing 10% glycerol and 1% albumin (Meyer’s solution, used for fixing smears before staining) had been incorrectly labelled as Fructose and was used in the experiments by error. Nonetheless, the fortuitous discovery that glycerol and other agents could be used as cryoprotectant(s) to significantly increase spermatozoa survival was fundamental to further development and clinical translation of sperm cryobiology. The first calf born from frozen thawed sperm by artificial insemination was subsequently reported in 1952 (Polge, 1952) and the use of frozen thawed sperm from a person who was dead at the time of inseminating his wife was reported shortly after, in 1953 (Bunge and Sherman, 1953).

Mainstream cryopreservation of human spermatozoa followed agricultural application and was first introduced in the 1960s (Sherman, 1973). The inaugural human sperm banks were in Iowa, USA and Tokyo, Japan (Park, 2018). However, fertility treatment using donor sperm
led to public outcry, levelling accusations of adultery and creation of illegitimate children, as well as political and religious objections, so clinics redirected their focus to fertility preservation for men. Political and sociocultural change in the 1970s facilitated subsequent acceptance of donor insemination, however most treatment at that time used freshly donated unprepared semen. Development of semen processing techniques, either by swim up or density gradient centrifugation (DGC), was a collateral benefit from the development of IVF, but it was the discovery (circa 1984) that semen could transmit the HIV virus (Stewart et al., 1985) and the introduction of statutory quarantine requirements with restrictions on the use of fresh semen that helped sperm banks finally gain acceptance. Fertility treatment using screened quarantined cryopreserved donor sperm is now widely available and highly commercialised. The human sperm bank market is valued at approximately $4.33 billion (2019) and is expected to reach around $5.45 billion by 2026, expanding at a compound annual growth rate of 3.3% (https://dataintelo.com/report/sperm-bank-market/).

**Autologous sperm cryopreservation**

Beyond sperm donor programmes, sperm banking is of fundamental importance because it allows cryopreservation and autologous use of gametes at a later date. It is therefore highly relevant to patients facing gonadotoxic treatment. Improvements in cancer detection and treatment has resulted in prolonged survival and cure of patients (Dal Maso et al., 2019). As such, the emphasis of cancer care is not solely focussed on survival and has shifted to include consideration of quality of life, including fertility for men (and women) of reproductive age. The negative effect of cancer and its treatment on spermatogenesis is well established (Meistrich, 2013). Although a large proportion of men undertaking cancer treatment retain or regain spermatogenesis and achieve natural conception (Brydoy et al., 2005), sperm cryopreservation is undoubtedly the best pre-treatment insurance, offering an opportunity of future fertility. But sperm cryopreservation has a reach beyond orchidectomy, chemotherapy and/or radiotherapy as it also offers reproductive potential for transitioning transwomen as well as those undertaking treatment for benign medical conditions where treatment is likely to affect spermatogenesis, and/or reproductive function (Rozati et al., 2017).

Sperm cryopreservation for men with severely impaired spermatogenesis of both known and unexplained aetiology, usually in advance of ART, is arguably the commonest reason for short-term freezing sperm. Indeed, a recent audit of sperm cryostored at Ninewells Assisted Conception Unit, Dundee (n=279; excluding samples in long term storage for fertility preservation), found that 50% of samples stored were from men with severe oligozoospermia, as a precaution in case of azoospermia on the day of egg collection. Of the remaining samples, 27% (n=75) were stored following surgical sperm retrieval (SSR), 11% (n=31) cryostored samples were from those likely to be away at the time of treatment including working offshore or military deployment, 5% (n=14) samples stored were due to erectile dysfunction (ED) or where difficulties had been previously encountered or were
anticipated in producing sperm on the day of egg collection, 4% (n=11) samples were stored following medical induction of spermatogenesis for hypogonadotrophic hypogonadism or pituitary dysfunction and 3% (n=5) samples were stored for other reasons, including quarantine prior to surrogacy (Figure 1). Clearly, an uncertain prognosis for ongoing sperm production represents no pressing need for fertility preservation, yet cryopreservation prior to ART offers reassurance for those with severely impaired spermatogenesis in case of azoospermia on the day of egg collection. A retrospective study of 247 men who undertook sperm cryopreservation for impaired spermatogenesis prior to assisted conception reported 52% (34/66) risk of azoospermia in at least one ICSI attempt when at least one total sperm count was <100,000 versus 3% (5/181) when all counts were \( \geq 100,000 \) (Montagut et al., 2015). Nonetheless, who and when to offer autologous sperm cryopreservation is not clearly defined and therefore practices vary. For example, sperm cryopreservation is offered to those with severely impaired spermatogenesis in our centre, with the majority of samples stored for those with sperm concentration <1 million/ml (Figure 2). However, although sperm cryostorage as back up for ICSI may be justified, it is worth noting that post-thaw samples are likely to have significantly lower sperm count and poorer total and progressive motility (Petyim et al., 2014). Cryosurvival factor (CSF) and is calculated using the formula:

\[
CSF = \frac{\text{% motile spermatozoa post thaw}}{\text{% motile spermatozoa pre freeze}} \times 100
\]

Despite numerous achievements in sperm cryobiology and widespread clinical use of cryopreserved sperm, the reality is that not all spermatozoa survive the freezing and thawing process, with post-thaw sperm concentration and motility up to 50% lower than respective pre-freeze values in humans (Mortimer et al., 2022). Various factors during the freezing process contribute to this phenomenon, mostly driven, either directly or indirectly, by ice formation and osmotic stress.

**Freezing sperm**

The main objective of freezing semen is to maintain sperm viability and function over an extended period of time. Given that all metabolic reactions and biological processes are suspended, cryopreserved sperm can theoretically be kept indefinitely. This is supported by reports of pregnancies following ART using semen frozen for at least several decades in both humans (Horne et al., 2004, Feldschuh et al., 2005, Szell et al., 2013) and farm animals (Salamon and Maxwell, 2000). However, semen is very heterogeneous and successful cryopreservation varies between species, between individuals of the same species, and even between ejaculates from the same individual.

Liquid nitrogen (LN\(_2\)) is an inert cryogenic fluid. All biological activity stops below the glass transition temperature of water (around -80 to -135°C) (Bojic et al., 2021). Spermatozoa are
routinely cryostored at -196°C in liquid nitrogen or vapour phase. However, human cells usually operate within an extremely narrow temperature range (36 - 38°C) and unprotected cooling and thawing of cells is not compatible with viability. Spermatozoa are very small cells with a relatively large surface area, and potentially less susceptible to cryodamage compared to larger cells. Nonetheless, the ability to induce and reverse low-temperature states in a controlled manner with minimal transition-related damage and destruction, specifically cold shock and chill damage above 0°C and intracellular ice formation as the temperature falls below freezing to -130°C, is critical to successful cryostorage and subsequent revival of cells. In practice, this is the central challenge of cryopreservation. To mitigate these effects, two protective actions must be carried out: selection of an appropriate cooling (and thawing) rate and use of cryoprotectant(s).

**Slow freezing**

Cryopreservation inflicts cellular damage largely as a result of uncontrolled transition between normal and low temperature states, specifically mechanical damage from dehydration and shrinkage of cells during freezing and/or rehydration and swelling upon thawing. Importantly, cell survival is influenced by both formation of intracellular ice as well as osmotic stress. If a sample is cooled too quickly then spermatozoa do not efficiently dehydrate and formation of intracellular ice results in membrane disruption and cell death. If cooling is too slow then osmotic stress arises from solution effect, due to the interaction of membrane proteins and high ion concentrations extracellularly and/or mechanical disruption of the cell membrane due to low cell volume. A transition zone (TZ), where negative effects of both intracellular ice (II) and solution effect (SE) are minimised, describes optimal cooling conditions for cryopreservation (Figure 3). This varies for different cells and different species. Conventional (slow) freezing is the usual approach for human sperm, where cooling to -196°C occurs over several hours by stepwise reduction in external temperature conditions (ideally achieving an initial cooling rate of 0.5-1°C/min to 5°C and then 10°C/min) (Whaley et al., 2021).

**Cryoprotectant Agents**

The addition of cryoprotectant agents (CPAs) to cryopreservation medium (CPM) aims to protect spermatozoa against both mechanical and ionic effects of dehydration and ice crystal formation, as well as stabilising the cell membrane (Elliott et al., 2017). CPAs may be permeating (low molecular weight compounds that pass through the cell, for example glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, propylene glycol) or non-permeating (higher molecular weight compounds that generally act to promote fast cellular dehydration, for example sucrose, polyvinlypyrrolidone (PVP), raffinose and trehalose). Permeating and non-permeating CPAs are usually used in combination. However, caution is required as permeating CPAs may be toxic and result in cell damage and death. The addition of CPAs also exerts osmotic stress by increasing the osmolarity of the surrounding medium,
resulting in sequential cell shrinkage and then swelling during permeation. Most protocols therefore require slow (dropwise) addition of CPM with continual mixing.

Glycerol is the commonest permeating CPA in commercially available CPM, usually in combination with sucrose or glucose as non-permeating CPA (Raad et al., 2018). CPM also contains other agent(s) to stabilise the plasma membrane, for example egg yolk, albumin, milk or glycine, which either alter the lipid composition of the cell membrane or directly interact with membrane lipids and proteins. Chelators, for example ethylenediaminetetraacetic acid (EDTA) and citrate, are included to stabilise intracellular calcium concentration, as well as compounds to inhibit lipid peroxidation and protect against oxidative stress. Lastly, CPM contains buffers to maintain pH stability, usually zwitterionic buffers, for example HEPES or TES, where the acid or base component is dipolar, as these are generally considered superior. Exact formulations of commercial CPM in clinical use are not readily available within the public domain, however composition is highly variable (Table 1).
Sperm cryodamage and oxidative stress

Although multifactorial, oxidative stress (OS) is an important mechanism underlying human sperm cryodamage. Induction of OS results from increased generation of reactive oxygen species (ROS) and nitrogen reactive species (RNS). Importantly, ROS such as hydrogen peroxide ($H_2O_2$), superoxide anions ($O_2^{-}$) and hydroxyl radicals ($OH^{-}$) can cause lipid peroxidation of the cell membrane and disruption of mitochondria, sperm DNA damage and as well as apoptosis and cell death (Martins da Silva, 2019). Spermatozoa are particularly susceptible to ROS-induced oxidation due to the abundance of polyunsaturated fats in the plasma membrane (Aitken et al., 2014). Lipid peroxidation alters cell membrane fluidity and permeability with loss of membrane integrity, as well as detrimental effects on motility (Baker et al., 2015, Nowicka-Bauer et al., 2018). Damage of cellular structures, including the acrosome and plasma membrane may also subsequently manifest as reduced ability to fertilise an egg (Bollwein et al., 2008). This may have implications for downstream clinical treatment, notably requiring the use of ICSI rather than IVF or intrauterine insemination (IUI) for some patients. Many studies have investigated the effects of supplementing CPM with antioxidants to neutralise ROS, both to minimise DNA damage and improve quality and/or preserve function of post-thaw spermatozoa. Both synthetic and natural antioxidant compounds have been added to CPM, with some showing noticeable and positive effects on semen characteristics both in vitro and in vivo (Amidi et al., 2016, Hezavehei et al., 2018). Among others, myo-inositol is one of the most powerful naturally occurring antioxidants that has been reported to be effective in improving sperm quality and motility when used both in vivo (Montanino Oliva et al., 2020, Santoro et al., 2021) and in vitro (Ghasemi et al., 2019, Artini et al., 2017). Myo-inositol appears to be helpful in protecting against OS during cryopreservation (Azizi et al., 2022) and improves sperm characteristics (vitality and motility) when added after thawing ($p < 0.05$) (Ponchia et al., 2021).

Antioxidants can be divided into two categories (enzymatic and non-enzymatic) according to chemical structure. Enzymatic antioxidants include glutathione peroxidase (GPx), catalase and superoxide dismutase (SOD). They work by breaking down and removing free radicals, in the presence of co-factors such as copper, zinc, manganese and iron. Non-enzymatic antioxidants work by interrupting free radical chain reactions and include vitamin C, vitamin E, carotenoids, glutathione, taurine and melatonin as well as co-factors selenium and zinc. A recent systematic review and meta-analysis showed overall positive effect of antioxidants on progressive (but not total) sperm motility and viability, as well as a reduction in DNA damage and $H_2O_2$ production during the freeze-thaw process (Bahmyari et al., 2020). However, whilst data support addition of antioxidants to CPM, they do not commonly feature in commercial preparations.

ROS production is associated with damage to sperm DNA and chromatin structure as well as alterations to DNA methylation (Khosravizadeh et al., 2022). Notably, spermatozoa from infertile men appear to be more vulnerable to cryodamage compared to gametes from fertile men (Donnelly et al., 2001). Yet the wider implications of cryopreservation on sperm...
quality and function are not completely understood. Cryopreservation has been reported to affect the expression of key genes related to fertility potential, specifically SNORD116, PWSAS and UBE3A, which may have implications for offspring conceived using cryopreserved sperm (Valcarce et al., 2013). Gene and protein expression, mRNA stability and epigenetic content of spermatozoa may be affected by the freeze-thaw process, however, the understanding of the transgenerational effects of cryostored sperm is limited (Hezavehei et al., 2018).

Cryostorage: methodology and practical aspects
Semen samples are collected by masturbation after 2 - 5 days abstinence and liquefied at room temperature. Semen analysis is performed prior to processing for cryopreservation. Techniques of human sperm cryopreservation are described in detail elsewhere (Mortimer et al., 2022). The main debate appears to be whether to prepare samples, usually by DGC, and then freeze, or to freeze unprepared samples, with subsequent preparation by DGC, which enables simultaneous removal of CPM prior to use. Freezing unprepared samples allows exploitation of the protective antioxidant properties of seminal plasma (Donnelly et al., 2001, Grizard et al., 1999) and is our preferred approach (see best practice protocol for sperm cryopreservation; Appendix 1). Antioxidants naturally found in semen include vitamins C, E, B9 (folic acid), trace elements zinc and selenium and micronutrients such as carnitines and carotenoids (Martins da Silva, 2019), which may be beneficial given OS associated with freezing and thawing sperm (Kumar et al., 2019). Notably, seminal plasma total antioxidant capacity is generally lower in men with impaired spermatogenesis (Roychoudhury et al., 2016, Gholinezhad et al., 2020) so the potential advantage of freezing unprepared samples may be smaller in this patient group. Conversely, several small studies have reported higher post-thaw total and progressively motility as well as vitality for semen samples prepared prior to freezing, suggesting that minimal post-thaw processing might be advantageous, particularly for donor sperm samples where count and motility is good (Brugnon et al., 2013, Androni et al., 2021).

Use of commercially available CPM, supplements and consumables must conform to pharmaceutical standards. Medical devices require CE (Conformité Européene) marking before they can be sold in European Economic Area (EEA). CE marking signifies products that have been assessed to meet safety, health and environmental legislative requirements. This has been replaced by UKCA (UK Conformity Assessed) in UK and is similar to the UL mark in USA and Canada. CPM should be CE-marked or equivalent, certified as pyrogen-free and with no known sperm toxicity. UK practice requires patients to be screened for infectious diseases prior to cryostorage of gametes for autologous use (Hepatitis B (HBsAg and anti-hepatitis B core), Hepatitis C and HIV 1&2) and to complete necessary legal consents (HFEA https://portal.hfea.gov.uk/media/1756/2021-10-26-code-of-practice-2021.pdf).
Cryostorage tanks are insulated cylindrical containers made from stainless-steel or aluminium. The largest high-capacity tanks can hold up to 500 litres LN\textsubscript{2} and potentially thousands of samples. They usually feature temperature alarms and LN\textsubscript{2} sensors and have an autofill function from secondary LN\textsubscript{2} tanks when levels are detected to be low. Small tanks can hold 30 – 60 litres LN\textsubscript{2} and are usually manual, rather than auto-fill. Both types of tanks have a vacuum between the outer shell and inner storage chamber, to minimise loss of LN\textsubscript{2}, thus allowing the tank to maintain a nearly steady state for up to several weeks if filled properly and not opened. The inner storage chamber is suspended inside the outer shell by the neck. Cold is conducted from the bottom of the tank to the neck, so even a small amount of LN\textsubscript{2} in the tank will keep the contents frozen. Because of the risk of asphyxiation when used or stored in poorly ventilated areas, LN\textsubscript{2} tanks must be located in a well-ventilated area with monitored oxygen levels.

Cryopreserved specimens may be stored in either liquid or vapour phase. When submerged in LN\textsubscript{2}, cryopreserved samples are securely maintained at a stable temperature of −196°C. The temperature cannot rise abruptly, and there is therefore time to rescue and relocate samples in the event of catastrophic tank failure. However, most micro-organisms, including viruses, can survive in LN\textsubscript{2} (Piasecka-Serafin, 1972, Bielanski et al., 2000). Vapour phase storage is therefore commonly used for virus-positive patient samples to prevent the risk of viral transmission by contamination of LN\textsubscript{2} in direct contact between samples (Bielanski, 2005). Vapour-phase nitrogen is also utilised in shipper tanks and large bulk tanks used at long-term storage repositories. The vapour in which samples are stored is typically −150°C or lower. Importantly, vapour storage tanks with an auto-fill function need to be continuously monitored, as a tank failure will lead to a relatively rapid rise in temperature and insufficient time for personnel to respond before gametes are compromised.

Accredited embryology and andrology laboratories that provide freezing of gametes and embryos are required to check tanks three times per week and/or have continuous monitoring via a level probe with alarms that are tested at least quarterly to ensure that cryostored samples are maintained under stringent conditions. Other essential requirements include having robust laboratory protocols, as well as personnel, knowledge and experience relating to best practice for safe and reliable cryostorage of samples (including safety data sheets and signage display), provision of appropriate training, adequate personal protective equipment (PPE) and enough LN\textsubscript{2} supply for emergencies (Practice Committees of the American Society for Reproductive Medicine et al., 2020).

**Vitrification of sperm**

Vitrification involves ultra-rapid cooling and warming rates to prevent intracellular ice formation. It is certainly the superior approach for egg and embryo cryopreservation (Cobo and Diaz, 2011, Rienzi et al., 2017), and appears to be advantageous compared to conventional freezing methods for human sperm in terms of CSF (Li et al., 2019). Although
the first reports of clinical outcomes using vitrified sperm were published a decade ago (Isachenko et al., 2012, Sanchez et al., 2012), vitrification remains a relatively unexplored methodology with only a few studies showing its efficacy in male gametes. Sperm vitrification appears to be simple and avoids/minimises the need for cryoprotectant (Aizpurua et al., 2017, Shah et al., 2019, Schulz et al., 2020). However, vitrification of sperm has limitations, not least because a very small volume is required to achieve the required cooling rate, which therefore makes cryopreservation of ejaculated semen less feasible. Single sperm vitrification has also been described (Liu and Li, 2020, Maleki et al., 2022) and may be particularly valid for patients with significantly impaired spermatogenesis and limited numbers of spermatozoa.

One legitimate concern with vitrification methodology regards direct contact of gametes with liquid nitrogen, with inherent risk of microbial contamination. Recently, new carriers have been developed, for example SpermVD (Berkovitz et al., 2018), to optimise freezing protocols and control volume and speed of cooling, as well as different strategies to minimize the risk of contamination. In summary, although sperm vitrification has not yet been applied in routine sperm cryopreservation, its potential as a procedure is growing (Tao et al., 2020). In due course vitrification may become a faster alternative method of sperm cryopreservation with significant benefits to fertility clinics in regard to simple equipment and easy methodology.

**Thawing sperm**

Following sperm cryopreservation, a ‘test-thaw’ is usually performed to assess sperm survival. The results of the test-thaw should be examined prior to removing straws from cryostorage, to determine the appropriate number of straws required for treatment. For extremely poor testicular samples, the patient may benefit from the preparation of multiple straws to maximise the quality of sperm used during the ICSI procedure. Conversely, for IVF, a single straw may be sufficient for treatment providing sperm survival is high. Cryoprotectant should be removed quickly when thawing sperm (see best practice protocol for sperm thawing; Appendix 2). This is typically achieved by diluting the sample with culture media. However, there is a significant risk of cell damage and death due to uncontrolled water diffusion and osmotic shock if a large volume of media is added. As such, media should be added to the frozen-thawed sample drop-by-drop, with the aim of gradually establishing an osmotic equilibrium between the intracellular cytosol of the spermatozoa and the extracellular environment. Samples are then washed by centrifugation. When preparing frozen-thawed ejaculates with a low spermatozoa concentration, the samples should be washed at a relatively high speed (800 x g) to maximise the concentration of spermatozoa in the final preparation. Conversely, testicular sperm are fragile, and the sample should be centrifuged at a low speed (≤200 x g) to prevent damage.
How long should we freeze sperm for?
Recent UK legislative changes allow sperm storage for up to 55 years. Whilst longterm storage of sperm has no impact on clinical outcomes (Huang et al., 2019), utilisation of cryostored sperm is notoriously low, although relatively few studies have reported this. A 15-year cryopreservation program involving 776 men with a diagnosis of malignancy who were referred for semen cryopreservation before proceeding with chemotherapy and/or radiotherapy reported 5.2% utilisation (Ragni et al., 2003); epidemiological analysis of sperm cryopreservation for male patients with cancer at the University of Pennsylvania revealed less than 5% of patients utilised their specimens for reproductive purposes (Chung et al., 2004); an evaluation of sperm cryopreservation services for cancer patients found that 4% patients utilised their specimens for reproductive purposes (Chang et al., 2006). These three studies were from European, American and Asian centres respectively, yet findings are very comparable. Similarly, a retrospective study of 545 patients suffering from cancer or benign diseases over a decade (January 2008 and July 2018) reported 5.3% utilisation (Vomstein et al., 2021). Notably, a retrospective review of 1442 sperm samples cryopreserved for fertility treatment (IUI, IVF/ICSI) as well fertility preservation related to cancer treatment or military deployment, reported overall utilisation rates of 19.3% (Machen et al., 2018). This study population is arguably much more representative of all those presenting for sperm cryostorage but again highlights the disparity between numbers of samples cryostored and those used. Clinical provision of sperm cryopreservation for impaired spermatogenesis thus raises significant practical challenges in terms of space and facilities required, as well as costs for monitoring and audit as well as administrative oversight.

Conclusions and future perspectives
Sperm cryopreservation for impaired spermatogenesis is an important, and effective, part of fertility management. Nonetheless, cryodamage following slow freezing has detrimental effects on sperm quality and function post-thaw. We need better understanding of the cellular and molecular changes that occur during freezing, and the mechanisms resulting in cryo-injury, to optimise of sperm cryopreservation. Alternatively, sperm vitrification, may offer a better option, particularly for men with significantly impaired spermatogenesis. Although cryopreserved spermatozoa have been used in fertility treatment for decades, transgenerational effects are uncertain and long-term follow-up studies of offspring conceived using cryopreserved spermatozoa should be performed to fully assess biological safety.

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Author contribution statement
SMDS conceived and wrote the manuscript. GH wrote best practice protocols. GH and SMDS edited the manuscript. Both authors approved the final version.

References


**Figure legends**

**Figure 1** Audit of sperm samples cryostored at Ninewells Assisted Conception Unit, Dundee excluding samples in long term storage for fertility preservation (n=279). 50% samples stored due to significantly impaired spermatogenesis (n=140).

**Figure 2** Sperm samples cryostored as treatment back up at Ninewells Assisted Conception Unit, Dundee (n=140). All samples cryostored due to varying degrees of significantly impaired spermatogenesis. The majority of samples were stored where sperm concentration <1 million/ml (75%; n=105).

**Figure 3** Cryodamage and cell survival during cryopreservation. **A** High cell damage due to solution effect (SE) when cooling rate is slow (dotted line). High cell damage due to intracellular ice (II) when cooling rate is high (dashed line). **B** Overall cell survival (solid line). Transition zone (TZ), where negative effects of II and SE are minimised, describes optimal cooling conditions for cryopreservation (green shaded area).

**Table 1** Composition of selected commercial CPM in clinical use (2022)

**Appendix 1** Best practice protocol for sperm cryopreservation
Appendix 2 Best practice protocol for sperm thawing
Table 1

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<td>+ (D,L)</td>
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<tr>
<td>Calcium chloride</td>
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<td>(dihydrate)</td>
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<td>Potassium chloride</td>
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<td>Sodium Chloride</td>
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<td>Magnesium chloride</td>
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<td>Potassium phosphate</td>
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<td>Physiologic salts</td>
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<td>Dextrose monohydrate</td>
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<td>Fructose</td>
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<td>Gentamicin</td>
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<td>Phenol red</td>
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*Exact amount/composition not specified by the manufacturer
Figure 1 Audit of sperm samples cryostored at Ninewells Assisted Conception Unit, Dundee excluding samples in long term storage for fertility preservation (n=279). 50% samples stored due to significantly impaired spermatogenesis (n=140).

338x190mm (54 x 54 DPI)
Figure 2 Sperm samples cryostored as treatment back up at Ninewells Assisted Conception Unit, Dundee (n=140). All samples cryostored due to varying degrees of significantly impaired spermatogenesis. The majority of samples were stored where sperm concentration <1 million/ml (75%; n=105).
Figure 3 Cryodamage and cell survival during cryopreservation. A High cell damage due to solution effect (SE) when cooling rate is slow (dotted line). High cell damage due to intracellular ice (II) when cooling rate is high (dashed line). B Overall cell survival (solid line). Transition zone (TZ), where negative effects of II and SE are minimised, describes optimal cooling conditions for cryopreservation (green shaded area).
**Best practice protocol for sperm cryopreservation**

The same basic method of sperm cryopreservation may be used for all sperm samples regardless of source and maturity (fresh ejaculate, retrograde ejaculate, testicular spermatozoa). Ideally, cryopreservation is performed on neat semen samples. In case of very low sperm concentrations, it is advisable to concentrate the sperm before freezing. In case of very high sperm concentration, sperm can be diluted with buffer medium before freezing, if preferred.

**Materials:**

- Freezing tank with Liquid Nitrogen
- Class II safety cabinet
- Incubator
- Centrifuge
- Racking
- Counting chamber
- Straw Labeller
- Straw labels
- Straw sealer
- Sterile pipette tips
- Specimen pot
- Slides and coverslips
- Pipette
- Sterile long-form Pasteur pipettes
- Sample preparation and waste tubes
- Sperm straws – CBSTM high security sperm straws 0.5ml
- Sterile filling nozzle CBSTM high security straws
- Straw filling device (unit-specific)
- FertiPro SPF: SpermFreeze™

**Method**

1. Place the specimen pot in a class II safety cabinet and leave to liquify at room temperature for 30 minutes.
2. Assess sample volume, appearance and liquefaction (not applicable to TESE samples). Sperm concentration, motility and morphology should also be determined and recorded.
3. Cryopreservation media should be well mixed and equilibrated to room temperature before use.
4. Add the required volume of cryopreservation media according to manufacturer instructions. For example, SpermFreeze™ (FertiPro) is a ready-to-use HEPES buffered cryopreservation medium and requires 0.7ml medium for 1ml sperm.
5. Add cryopreservation media to the sperm sample in a drop-wise manner, with each drop followed by gentle agitation; this action prevents sperm damage through osmotic shock.

6. Following the addition of cryopreservation media, leave the sample to equilibrate at room temperature for 10 minutes.

7. Following equilibration, aspirate (using syringe and adapter or aspirator) the sperm/cryopreservation media mixture into a CBSTM high security sperm straw until the sperm mix reaches the hydrophobic plug. This seals the straw whilst leaving an airspace in the lower part of the straw to allow for expansion during freezing. Take care to avoid contaminating the outer surface of the straw.

8. Remove the filled straw from the micro-aspirator/syringe attachment. Remove the filling nozzle. Heat-seal both ends of the CBSTM high security sperm straw using the sealer as per manufacturer’s instructions and place the straw to one side within the safety cabinet. Repeat the process until all straws are filled and sealed.

9. Shake to move the air-bubble to the centre of the straw.

10. Transfer straws quickly into liquid nitrogen and store at -196 °C.
Best practice protocol for sperm thawing

Materials
- Class II safety cabinet
- Centrifuge
- Racking
- Counting chamber
- Sterile pipette tips
- Slides and coverslips
- Pipette
- Sterile long-form Pasteur pipettes
- Sample preparation and waste tubes

Method
- Before use, remove as many straws or cryovials as required from the liquid nitrogen or vapour tank and place them immediately in tap water for 5 minutes (room temperature or 37 °C). Due to the large surface area-to-volume ratio of the cryopreservation straws, the contained sample will thaw rapidly.
- After complete thawing, cut off the end of the straw with sterile scissors and load the insemination device (for therapeutic use) or expel the contents to determine post-thaw motility (to check the freezing process). The straw contents are emptied into a sterile tube by cutting below the hydrophobic bung at the top of the straw with a pair of sterile scissors. Any residual sample remaining in the straw is used to create an examination slide for assessing semen parameters.
- Remove the cryoprotectant by adding culture medium before centrifugation for 10 minutes at 500g. Remove the supernatant and dilute the sperm pellet in culture medium to the appropriate volume.
- If necessary, use sperm preparation techniques after thawing the semen to eliminate dead sperm cells and debris. Dilute the concentrated sperm in a suitable insemination medium.