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Chapter

Rare Sperm Freezing

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Abstract

Gamete cryobanking has been widely incorporated in present assisted reproductive technology (ART). Preserving male gametes for future fertility is considered to be an easy and accessible way to insure one's reproduction. Despite the fact that the method could not secure success, sperm freezing could be the only chance to father biological offspring. In cases when severe male factor (SMF) infertility is diagnosed (retrograde ejaculation, virtual azoospermia, obstructive azoospermia, cryptozoospermia) and providing fresh semen samples for assisted reproduction may alter chances to achieve pregnancy, rare sperm cryopreservation could contribute for conceiving. Isolation, selection and cryopreservation of single sperm cells from semen samples is a challenging procedure. Different approaches and devices could be used in order to extract utmost spermatozoa. Aiming to highest cryosurvival rates sperm freezing protocols should be carefully considered. For some men, rare sperm cryopreservation might be the only alternative for parenting biological offspring. Thus, the latter technique should be widely discussed, developed and practiced in assisted reproduction.

Keywords: cryopreservation, fertility preservation, single sperm selection, sperm sorting devices, cryptozoospermia, sperm genetics

1. Introduction

Gamete cryobanking has been considerably incorporated in present assisted reproductive technology (ART). Preserving fertility through banking is an accessible and relatively reliable procedure that gives opportunity for men to parent their own biological child. Most of the clinics providing fertility treatment have their individual cryobanks and offer fertility preservation counseling. Network structures for gamete and tissue storage have also been developed. Some of the affirmed ones would be the Danish network (www.rigshospitalet.dk), *Ferti*PROTECT ® (www.fertiprotect.com), German-Austrian-Swiss centralized and decentralized network between the countries, Oncofertility® Concortium (www.oncofertility.northwestern.edu) for knowledge exchange in the field of fertility preservation [1]. The complexity of fertility preservation generates necessity for close interaction between the patients, reproductive specialist, reproductive biologist, urologist, oncologist, etc. In order to provide accurate and prompt counseling and treatment, each clinical case should be considered in full and timely.

Retrieving, freezing, storage and use of human oocytes, spermatozoa, embryos and ovarian and testicular tissue has been executively studied and explored in assisted reproduction (AR). Cryopreservation presents remarkable advance to men and women who have decided to postpone fertility. It turns out to be a safety plan for patient with subfertility and certain physiological or psychological conditions.

For patients with forthcoming cancer treatment it could be the only chance to have their own biological child.

Nowadays cryopreservation and cryobanking is inseparable branch to assisted reproduction and fertility preservation treatment.

2. Historical preview of sperm cryopreservation

First attempts to preserve human spermatozoa dates back to 1776 when Lazaro Spallanzani studied sperm cryopreservation by cooling it in snow. It was 1949 when Polge, Smith and Parkes discovered glycerol to be effective in protecting spermatozoa exposed to low temperatures [2]. This discovery, alongside with the first reports for achieving pregnancy by frozen and thawed spermatozoa in 1953 by Dr. Jerome K. Sherman [3], led to constant development and improvement of sperm freezing protocols and devices. In 1972 a slow freezing protocol, developed by D. Whittingham, S. Leibo, and P. Mazur, was introduced. Slow cooling with temperature drops in the range of 0.3 to 2 degrees Celsius per minute and consequent slow warming (4 to 25 degrees C° per minute) was performed. This protocol, applied to mouse embryos, resulted in 65% pregnancy rate and 40% full term pregnancy [4]. Recent challenge in cryobiology was freezing spermatozoa from strains of genetically engineered mice. A novel method using a cryoprotectant composed of 18% raffinose pentahydrate and 3% skim milk was presented [5, 6].

Not only protecting sperm cells at low temperatures, but preserving their structural (morphological), kinetic and functional characteristics is at aim when freezing semen samples. It is well known that cryopreservation has deleterious effect on sperm cells.

Although spermatozoa are relatively small in size and have large surface, cold shock and ice formation could damage different cell structures and organelles, as most affected structures are the plasmalema, acrosome and the tail [7]. Changes in membrane organization and permeability, formation of reactive oxygen species (ROS) and consequent DNA damage as a result to freezing hinders normal sperm activity and functions [8].

Semen cryopreservation strives high quality of the preserved samples. Thus, retaining sperm motility and viability, membrane integrity and intact DNA in thawed samples, has been formed as priority when developing freezing protocols. Cryoprotective medium, containing various additives – fatty acids, proteins, antioxidants, serum, essential oils derived from plants, nanoparticles and others, are also used in sperm freezing procedures.

3. Conditions requiring sperm preservation

Diversity of health conditions and personal or lifestyle circumstances could necessitate semen cryopreservation.

3.1 Sperm freezing for cancer patients

Five most common cancers diagnosed in men are prostate cancer, lung cancer, colorectal cancer, bladder cancer and melanoma. Testicular tumors, relatively rare condition on a per-population basis, are the most common malignancy in men aged 20 to 35 years [9, 10]. According to The National Cancer Institute one in two men will be diagnosed with cancer during their lifetime. Encouraging data for approximately

1.8% decrease in cancer death for male patients was recently published [11]. Modern medicine and constant scientific research in the field are the key to increasing the chances for long term survival (5 and above years). Quality of life after cancer treatment and when in remission is of great importance. Unfortunately, cancer by itself and chemo- and/or radiotherapy treatment, have adverse effect over the process of spermatogenesis. For most cured patients, after healing, sperm production recovers to a certain level in time. When bilateral orchiectomy was performed or high dosage of radiotherapy (24–25 Gy) was administrated permanent loss of fertility is inevitable [12]. Introducing cancer patients to fertility preservation before the treatment is required, especially for adolescents and young adults (aged 15–39 years) [13].

3.2 Sperm freezing for patients with retrograde ejaculation and hypospermia

Ejaculatory disorders could lead not only to psychological distress but may be the reason for 0.2–3% of infertility incidence in the couple. Co-ordination between epididymis, vas deferens, prostate, seminal vesicles, bladder neck and bulbourethral glands is required for the proper course of ejaculation. Various pharmacological, neurogenic or anatomic factors, disrupting the contraction of the bladder neck, may lead to retrograde ejaculation. In such cases semen is refluxed in the bladder and blends with the urine [14, 15]. As the urine normally has slightly acidic pH levels (average value - 6.0) compared to pH 7.1–8.0 of semen, the fusion of these fluids and pH fluctuations has adverse effect on spermatozoa [16]. Sperm cells retrieved from postejaculatory urine could be proceeded for assisted reproduction. Strict protocols for urine alkalization prior the procedure are mandatory. In some patients retrograde ejaculation results in hypospermia (abnormally low volume of less than 1.5 ml of the semen sample). Congenital absence of seminal vesicles and vas deferens, blockage of the ejaculatory duct, sympathetic nerves damage, and bladder neck surgery, insufficient levels of testosterone and short abstinence periods could also be the reason for hypospermia.

Retrograde ejaculation and hypospermia are linked to poor sperm parameters even cryptozoospermia. Freezing spermatozoa for fertility preservation in order to secure ART procedure would benefit any patient diagnosed with the described conditions.

3.3 Sperm freezing for patients with diabetes

Diabetes, a chronic autoimmune disease, is known to have detrimental effect to male fertility and sperm quality. Erectile dysfunction, retarded ejaculation and retrograde ejaculation could be persistent in patients with diabetes type 1 or 2. Reduced sperm quality and sperm DNA integrity impairment are also consequences to this health condition [17]. As all of the aforementioned are to affect fertility, cryopreservation of spermatozoa should be considered.

3.4 Sperm freezing for patients with Y-microdeletion and genetic aberrations

Alterations in autosomal genes, specific mutations/deletions of several X- or Y-chromosome genes, microdeletions in the azoospermic factor (AZF) regions of the Y chromosome and chromosomal anomalies can cause spermatogenic failure and affect male fertility.

Aberration in numerous autosomal genes result in fertility disturbance:

SYCP3 (synaptonemal complex protein 3) - meiotic arrest and consequent azoospermia.

PLK4 (Polo-like kinase 4) – Sertoli cell only (SCO) syndrome.

NANOS1 (**Nanos C2HC-Type Zinc Finger 1)** – SCO syndrome and oligoasthenoteratozoospermia.

HSF2 (heat-shock factor protein 2) - idiopathic azoospermia.

TAF4B (**TATA-Box Binding Protein Associated Factor 4b) - azoospermia.**

ZMYND15 **(Zinc Finger MYND-Type Containing 15) - azoospermia.**

SPATA16 (**Spermatogenesis-associated protein 16)** – globozoospermia.

KHLH10 (**Kelch Like Family Member 10)** – oligozoospermia.

SEPT12 (septin 12) - oligoasthenozoospermia or asthenoteratozoospermia.

GALNTL5 (**Polypeptide N-Acetylgalactosaminyltransferase Like 5)** – asthenozoospermia.

AURKC (**Aurora Kinase C) -** large-headed polyploid spermatozoa or macrozoospermia.

Alterations in X-chromosome located genes and fertility disturbance:

TEX11 (**Testis Expressed 11)** - meiotic arrest and consequent azoospermia.

RHOXF1 and RHOXF2/2B (human reproductive homeobox (RHOX) genes) - severe oligozoospermia.

ANOS1 (**Anosmin 1)** - anosmin-1 is involved in the migration of neurons producing gonadotropin-releasing hormone (GnRH). The latter controls the production of several hormones triggered to sexual development before birth and at puberty.

USP26 **(deubiquitinating enzyme gene) -** nucleotide variations in fertile and infertile men.

TAF7L (TATA-box binding protein associated factor 7) – reduced sperm count and motility, abnormal sperm morphology [18–20].

Y-microdeletion and fertility:

Y-chromosome microdeletions (YCMs) are the most common known structural chromosomal abnormalities for spermatogenic impairment. As high as 25–55% of the patients with hypospermia, sperm maturation arrest and SCO syndrome and 5–25% of the patients with severe oligozoospermia or azoospermia are established to have YCMs [21].

Deletions occur in three specific subregion - AZFa, AZFb and AZFc of the AZF in the long arm of the Y chromosome. Deletions in these regions are associated with:

AZFa region partial removal - hypo-spermatogenesis.

AZFa region complete deletion - inhibits the production and maturation of germ cells; SCO syndrome.

AZFb region deletions - pre-meiotic spermatogenic arrest or SCO syndrome; azoospermia, oligozoospermia.

AZFc region partial deletions - hypospermatogenesis.

AZFc region complete deletion - SCO syndrome and alterations in spermatocyte maturation [22].

Different studies indicate highest incidence of Y-microdeletion in the AZFc region, followed by AZFa+b + c; AZFb+c; AZFb; AZFa; and partial AZFa region deletion [23–25].

Passing genetic impairment to the offspring should be of high caution when sperm freezing and consequent ART is discussed.

3.5 Sperm freezing for patients with severe oligoasthenoteratozoospermia (OAT) or cryptozoospermia

Cryptozoospermia or virtual azoospermia and severe OAT may be the consequence to states of distinct origin. Extremly low sperm count in the ejaculate can

occur for hormonal reasons, injuries, infections, varicocele, genetic abnormalities and improper descent of the testicle into the scrotum in newborn and infants. Lifestyle, occupational and environment factors have been proven to show adverse influence over male fertility. Obesity, alcohol consumption in regular and high potions, sedentary lifestyle are among the factors with high impact over semen parameters. As a prominent part of today's mode of life, stress should not be underestimated as it negatively affects male fertility. Occupational exposure to pesticides, chemicals, hormones, regular intake of enhancing drugs or therapeutic drug treatment may have an influence on the highly sensitive process of spermatogenesis. Environment radiation, tocsins, air and water pollution, phthalates, etc. are also linked to decrease in sperm count and motility as they may alter spermatogenesis [26–29].

In all groups under risk of infertility due to any of the preceding factors, freezing several sperm samples should be considered as a safety pool [30].

4. Sperm freezing protocols and efficiency

There are two main protocols for sperm freezing:

4.1 Slow freezing

The protocol for slow freezing of spermatozoa was suggested by Behram and Salewa in 1966 [31]. It is based on slow dehydration of the cells. Slow freezing could be performed either manually or by using programmed freezer. The sperm samples are cooled in a stepwise manner by lowering the temperature and adding cryoprotectants. Initially the temperature is lowered by 0.5°C/min and it should go down from room temperature to +5°C. The second step is to freeze the samples from +5°C to −80°C by lowering the temperature by 1–10°C/min. Finally the semen samples are transferred into liquid nitrogen (LN2) at −196°C.

Automatic freezers have been reported as reliable when freezing sperm is performed. One of the advantages of these devices is the perfect control over temperature changes as the process is performed through software. The three step protocol of slow freezing takes about 40 min [32].

In slow freezing, changes in lipid phase transition and increase in lipid peroxidation, consequent to saturation with cryoprotectants, could cause intracellular and extracellular physical and/or chemical damage to sperm membranes. Susceptible to cryopreservation induced damage are the viability, motility and the morphology of the post-thawed spermatozoa. Mitochondrial function, as well as DNA integrity, could be affected [33–36].

4.2 Rapid freezing: vitrification

Vitrification, as a method for cryopreservation, has been primarily used for cryobanking of oocytes and embryos. This method is based on direct exposure of the gametes to liquid nitrogen at −196°C. In comparison to slow freezing, where formation of intracytoplasmic ice crystals could damage different cell structures, during vitrification the liquid components of the cells set into a glass like amorphous solid and ice crystal structures are avoided (**Figure 1**) [37].

Regardless the protocol used for cryopreservation of spermatozoa, cryoprotective medium must be inset in order to reduce the stress induced while freezing or thawing cells.

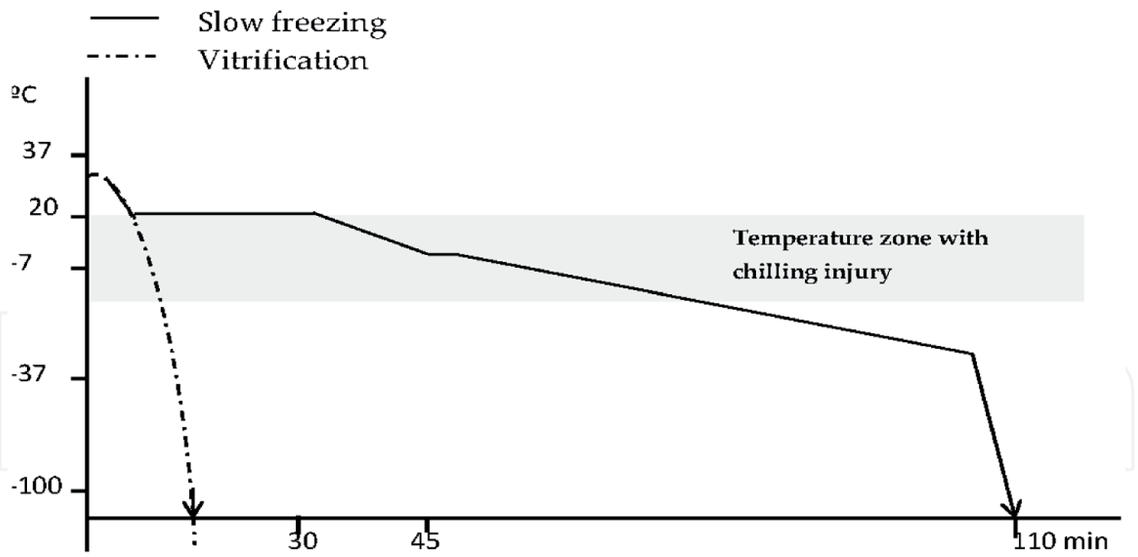

Figure 1.
Temperature changes in slow freezing and vitrification.

Permeable cryoprotectants can penetrate through cell membranes. They have intra- and extracellular activity. By forming osmotic gradient, water is ejected outside the cell, preventing it to form crystals. The cryoprotecting agents forms non-frozen channels in the medium in which sperm cells can be positioned while frozen. Glycerol, ethylene glycol, dimethyl sulfoxide (DMSO) and 1, 2 propanediol (PROH) are commonly used permeable cryoprotectants.

Non-permeable cryoprotectants cannot penetrate through the cell membrane. They can induce dehydration of the cells by increasing the concentration of extracellular solutes. Osmotic gradient is formed and the intracellular water is derived. Various sugars (raffinose, mannose, and trehalose) and proteins (lipoprotein, egg yolk) can be inset as *Non-permeable* cryoprotectants [38, 39].

5. Rare sperm freezing

Rare sperm freezing could be defined as a separate branch in sperm cryobiology. It has formed an important direction in the development of freezing protocols, methods and devices. The need for efficient freezing protocol for single sperm cells was evident at the very beginning, when pregnancies from epididymal and testicular sperm were reported [40–42]. In cases, where percutaneous epididymal aspiration (PESA) or testicular sperm extraction (TESE) is performed, freezing sperm cells would be of great benefit to the patient, as these procedures are traumatic and stressful to the organism. Most of the conditions described at *3. Conditions requiring sperm preservation* and poor semen quality are to request freezing of sporadic sperm cells.

Rare sperm freezing has some major advantages to standard protocols. In one hand this method gives chances for reliable fertility preservation in patient with severe male factor and sperm alterations. On the other hand, the successful freezing of single sperm cells and revolutionary methods such as intracytoplasmic sperm injection (ICSI), represent the opportunity for storage of larger quantity of samples for every men, as this premise increases the chances for fertilization of more oocytes retrieved in a cycle or secures larger number of ART cycles.

6. Rare sperm freezing protocols, carriers and devices

6.1 Biological carriers for rare sperm cryopreservation

6.1.1 Evacuated zona pellucida

Cryopreservation of single sperm cells in empty zona pellucida (ZP) was initially described in 1997 [43]. The biological carrier could be obtained by mouse or hamster pre-fertilization oocytes, human immature oocytes (e.g. germinal vesicle stage) prior fertilization, or embryos with abnormal fertilization and development after ICSI. The cumulus oophorus was removed via hyaluronidase and corona radiate was stripped by micropipettes. The oocyte was fixed with the holding pipette of the ICSI micromanipulator. By applying mechanical breach, chemical reagents (acidified Tyrode's solution) or highly focused laser beam, two small holes in the ZP were perforated. The cytoplasm of the oocyte must be aspirated and fully removed leaving the ZP empty of contents.

Sperm cells were obtained by centrifugation and placed in a droplet of 10% polyvinylpyrrolidone (PVP) solution. Using the ICSI needle each empty zona was injected with one to fifteen sperm cells. Slow freezing protocol and cryoprotective media of 8% glycerol solution in phosphate-buffered saline (PBS) and human serum albumin (3%) were preferred. The empty zonas were transfered in an individual sterile plastic straws of 0.25 ml. For easy location of their position they were situated between two small air bubbles.

Thawing of the biological carrier was implemented through exposure of the plastic straws to 30°C for 30 seconds in water bath. The content between the two air bubbles was extruded into droplets medium.

There are number of studies indicating that empty zona pellucida is an ideal carrier when it comes to freezing extremely small number of spermatozoa [44, 45]. Sperm recovery rate, compared to traditional freezing protocols, was higher, but motility recovery, DNA integrity and fertilization ability of sperm were similar in both methods [46]. From ethical point, it is important also to have an access to donated immature human oocytes and proper informed consent should be obtained (**Figure 2**).

Although this method shows high efficiency it is quite time and cost consuming and requires great precision with micromanipulation techniques and experience of the embryologist.

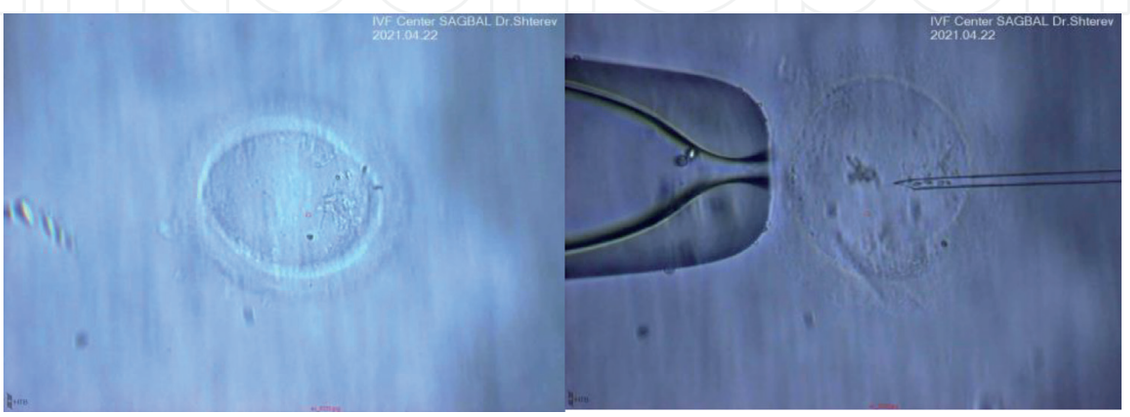

Figure 2.
Spermatozoa freezing in empty zona pellucida.

6.1.2 Volvox globator algae

Volvox gobator, a species of green algae of the genus *Volvox*, was described as a biological carrier for single sperm freezing and storage in 2004 [47]. The technique for loading rare sperm cells into the spherical algae is similar to the one used when freezing in ZP. Spermatozoa were injected in *Volvox globator* algae on a set of petri dish with media microdroplets and ICSI micromanipulator. In the pilot study, each Volvox sphere was loaded with eight male gametes. Cryoprotecting media containing 0.4% human serum albumin was supplemented. Each algae containing sperm was placed in a 0.2 mL plastic straw between two small air bubbles for easier location. Standard slow freezing protocol: 10 minutes at 4°C followed by 10 minutes of LN2 vapor and consequent submerging in LN2, was preferred.

Thawing of the biological carrier was performed through heating the plastic straws in a water bath at 25°C for 20 seconds. The content between the two air bubbles was extruded into droplets of medium. Sperm cells were subtracted through soft suction with the injection pipette at the micromanipulator set. The reported recovery rate (100%) and motility rate (at least 60%) were quite promising, but this method rises certain concerns. According to The United States *Food and Drug Administration (*FDA) and the European Tissue Directive regulations, there is no clear evidence that genetic material from the algae is not transferred and introduced into the oocyte with the injected sperm.

For the time being the use of non-human biological carrier in a clinical setting seems unacceptable. Still, there are countries with strict regulations prohibiting the destructive use of oocytes, and *Volvox globator* algae could be considered as an option for biological carrier when single sperm cryopreservation is nessesary [48].

Recently, non-biological carriers for rare sperm freezing, analogical to ZP and *Volvox globator* algae, were developed. Microcapsules composed of alginic acid, agarose or hollow hyaluronan -phenolic hydroxyl (HA-Ph) were tested with loading, freezing and thawing techniques adopted from the abovementioned biological carriers. First attempts were conducted in 2006, when sperm cells were frozen in polymerized alginic acid drops [49]. Although alginate is a non-toxic polysaccharide, the reported sperm motility after thawing was 20% lower compared to standard protocols. According to the study, decreased motility might be based on the adhesion of alginic acid to sperms surface.

Inactive and biologically sterile empty agarose microspheres of 100-μm in diameter were examined as carrier for the cryopreservation of one to ten sperm cells. The conducted post-thaw results in different studies show high recovery and motility rate (above 90% and above 80% respectively) and preserved membrane integrity of the cells [50, 51]. The hollow-core agarose capsule seems to be a promising substitute to ZP and *Volvox globator* algae.

Enzymatically fabricated hyaluronan (HA) microcapsules with thick membrane (30-μm) and 200 μm in diameter were also tested as carriers for cryopreservation of solitary spermatozoa. No differences according to recovery and motility rates after thawing of spermatozoa loaded into HA-Ph - microcapsules and ZP (95.5 vs. 93.9% and 13.6 vs. 15.1% respectively) were registered [48, 52].

Newly developed spherical analogues for rare sperm freezing would be of great benefit when ethical problems arise, and empty ZP could not be used for clinical or experimental application. No preliminary processing of the carrier is required and the procedure is less time consuming, but still highly qualified and trained embryologist as well as specialized technique is needed.

The inculcation of such promising non-biologically derived carriers in cryobiology needs further investigation and affirmation in regard to their safety and efficiency.

6.2 Non- biological carriers for rare sperm cryopreservation

6.2.1 Open-pulled straws

Open pulled straw (OPS) is a specially designed carrier for ultra-rapid vitrification. The tool was introduced in 1998 by Professor Gábor Vajta and it is considered to be highly efficient for single sperm cryopreservation. At considerably low risk of microbial contamination while in LN2, OPS could be incorporated for cryopreservation of very small volumes (1–5 μl), without the use of cryoprotectants. The reproductive cells are loaded in the end of the OPS by spontaneous capillary action when the straw is submerged into microdroplets of sperm suspension. The loaded straws, inserted into 90 mm straws, are hermetically closed and submerged in LN2 [53]. Preselection and loading of the sperm cells into the OPS could be performed via polar body biopsy (PBB) pipette [54]. In order to thaw the sperm cells, the outer straw is cut and the open pulled straw is drawn out. The tip should be immediately plunged into microcentrifuge tube containing media.

The OPS tool is of comparable efficiency to other systems and methods when rare sperm cells are cryopreserved. It is relatively easy to use and allows selection of sperm by its morphokinetic parameters prior cryopreservation.

6.2.2 Cryoloops

Cryoloops have been explored as a rare sperm freezing tool by Schuster et al. In 2002 [55]. Further investigation on nylon cryoloops with aspect to successful loading of preselected spermatozoa and cryopreserving oligozoospermic samples and surgically retrieved epididymal or testicular spermatozoa was conducted [56]. The open cryoloop should be dipped in small droplet of sperm suspension and placed into cryovial. Ultra-rapid freezing by either direct submersion in LN2, or 5 min of exposure to liquid nitrogen vapor prior submersion was performed. Standard slow freezing protocol could also be applied. Vitrification in cryoloops without additional cryoprotectants was also investigated and higher sperm motility compared to control group with cryoprotectants was reported (89.5 ± 7.1% vs. 77.5 ± 8.9%) [36].

Sperm samples were thawed by resuspendation in media immediately after the cryovial was taken out of the liquid nitrogen.

Sperm motility, viability, plasma membrane and acrosome integrity were assessed. Certain concerns according to acrosome damage and sperm cryo-capacitation after thawing have been arisen [57]. Cryo-injury is common consequence to different freezing protocols and cryoprotective media. When such small numbers of spermatozoa are frozen any structural or functional damage to the cell could be crucial to the overall treatment outcome.

This method would be convenient for sporadic sperm freezing due to its simplicity. It also provides a set of samples that could be used in multiple ART cycles for ICSI procedures. Cross-contamination should be considered as the cryoloop system is opened and allows liquid nitrogen flow.

6.2.3 Cryopreservation in microdroplets

Ultra-rapid cryopreservation of rare sperm cells in microdroplets results in high post thaw total motility and progressive motility rates compared to slow freezing technique. Sperm DNA fragmentation index (DFI) was also comparable to stated values in the fresh sample. On contrary, when standard slow freezing was conducted, DFI values increased significantly after cryopreservation in the post-thawed samples [58]. This method was adopted and resembles oocyte and embryo vitrification.

Suspension, obtained by the mixture of proceeded sperm and cryoprotective media (1: 1) added by drops in every 30 seconds, was prepared. Modified French mini straws, cut in half from the center to the end by its length, were loaded with 2 μl droplets of the suspension after it was equilibrated at room temperature for 10 minutes. Each hemi-straw (HS) was loaded by the extents of the open gutter with 10–15 droplets placed at equal distance of 2 mm in-between. The HS was placed in 0,5 ml straw and was secured through the two-stage stoppers which it has. After short exposure to LN2 vapor the straws were plunged into liquid nitrogen. Droplets carrying sperm cells were derived by gentle struck of the HS against the bottom of 1.5 ml Eppendorf tube. The samples were incubated at 37°C for 15 minutes. No additional centrifugation for enrichment of the sample was necessary. Washing the cryoprotective media was performed for the selected spermatozoa in droplets of the ICSI dish before sperm injection. This seems to be quite convenient when poor semen samples are frozen, as reduction of numerous centrifugation and washing steps increases the chance for higher number of sperm cells retrieved after thawing.

6.2.4 Cryotop

Cryotop, mainly used as embryo freezing and storage commercialized tool, has been adopted as a carrier for sperm vitrification. Different studies have been conducted in order to establish the most efficient protocol when Cryotop as a carrier is used. The most efficient protocol for the vitrification of rare sperm cells was setting 1 μl droplet of sperm suspension and sucrose as cryoprotectants on the strip. The Cryotop was immediately transferred over LN2 vapor (2 min at −120°C) and then plunged into liquid nitrogen. Reported recovery and motility rates after thawing sperm cells retrieved from the testes were 95% and 42.1%, and for single sperm cryopreservation obtained from ejaculates – 90% and 44.4% respectively [59]. Freezing semen samples on Cryotop and without cryoprotectants show higher viability after thawing and lower damage to DNA integrity compared to samples frozen with sucrose as adjuvant [60].

Cryotop is efficient tool for cryopreservation of embryos and rare sperm samples. It rises no ethical issues as it is of non-biological origin. The tool has been implemented in cryobiology and it has been randomly used resulting in successful pregnancies. In recent years, similar devices, based on knowledge cumulated from Cryotop examination, were developed. Moreover, due to its great performance, Cryotop has been used as a measure tool for newly developed sporadic sperm cells freezing devices and their efficiency [61].

6.2.5 Cell sleeper

Freezing small number of spermatozoa is still a challenge for modern cryobiology. In order to find not only the most reliable and efficient, but a carrier easy to use and relevantly less time consuming, different devices were fabricated, tested and compared.

Cell sleeper is a novel device with comparatively small use in practice. It is constructed of an inner tray as a sperm sample carrier and an outer vial. The system is closed and when the tray is placed inside the vial they are sealed together by a screw cap [62]. The device could be used as a cryopreservation carrier for preselected spermatozoa derived from ejaculates or from testicular tissue. Sperm cells were transferred from the proceeded samples in a microdroplet of freezing media on the tray of the Cell sleeper through ICSI needle. After placing the tray inside the vial and sealing it through the cup, the vial was positioned over LN2 vapor (2.5 min at −120°C) and then sunken into liquid nitrogen.

For thawing of the sample, the vial was warmed at room temperature for 1 minute. Afterwards the tray was drawn out of the vial and transfered in a petri dish then covered with oil. Following incubation period (37°C for 2 minutes) the recovery and motility rates were observed. Sperm recovery and motility rates of 94% and 56% were stated. Pregnancies were reported for both – ejaculated and retrieved from testicular tissue sperm cells [62, 63].

This device needs further investigation, but the comparatively large volume of the drop placed in the tray inevitably leads to time consuming search for the sperm cells. Since time for micromanipulation of the oocytes is important matter the latter could be considered as great disadvantage of the Cell Sleeper device.

6.2.6 Polydimethylsiloxane (PDMS) chip

Microfluidic devices for gamete handling have been incorporated in ART more than 20 years ago [64]. Since the initial study of the technology, the microfluidic systems developed specifically for sperm investigation, selection and cryopreservation, have been upgraded, improved and widely used in the clinical practice.

Polydimethylsiloxane (PDMS), silicone elastomer, chips for cryopreservation of single spermatozoa without cryoprotectants have been thoroughly investigated. Microfluidic two-layered chip was fabricated. The upper layer has two separate openings – inlet, transfusing into microchannel, and outlet. The smooth bottom layer is connected through plasma to the upper one. Different height of the microchannel were examined. For ultra-rapid freezing of rare sperm cells without cryoprotective media, best results were obtained when 10 μm height microchannel was tested [65].

Before cryopreservation the ejaculate should be processed by sperm gradient technique. The semen sample was loaded in the microchannel of the sterilized chip through the inlet. Approximately 5×10^{-3} μL medium containing 1000 sperm cells can be injected in the 10 μm channel by needle connected to micro-injector. The whole chip is covered in silver paper, so direct contact with liquid nitrogen could be avoided.

The chip containing sperm cells was thawed at 37°C for 10 min. The content was ejected in a culture petri dish through the outlet by applying pressure of a syringe connected with the input. Sperm viability, motility, DNA and acrosome integrity in post-thawed samples were researched. Comparison for PDMS chips with 10 μm height microchannel, where samples were frozen with ultra-rapid cryoprotectants-free protocol, and samples frozen through conventional slow freezing protocol was conducted. The compared post-thaw parameters for the investigated parameters were of comparable values. The most essential advantage of the on chip ultra-rapid cryoprotectants-free cryopreservation is the lack of cytotoxic cryoprotective media. The thawing of the sample, as no cryoprotectants were added, is simple and time and consumable saving [66].

6.2.7 Sperm vitrification device (sperm VD)

Sperm vitrification device (Sperm VD) is a novel tool specially designed for cryopreservation of small number of spermatozoa in microdroplets [67]. The device could be exclusively helpful when sperm cells are retrieved by means of TESE. Each Sperm VD plate carrier has three wells for placing sperm cells. Droplets of mixture 1:1 of cryoprotectants and washing media are placed individually for each well of the Sperm VD plate. Sperm VD should be transferred in a petri dish covered with oil. Using the injection needle of the micromanipulator, spermatozoa were transferred from neighboring droplets (PVP/washing media) into the droplets

of the wells of the Sperm VD. As cryoprotective media has negative influence on sperm cells it is crucial to minimize the time of exposure to the media before freezing the sperm. Substantial freezing should be done within 10 minutes past the first sperm cell transfer [68]. After loading each well of the Sperm VD plate it is transferred in pre-cooled cryotube over liquid nitrogen. The tool should be handled with tweezers and the excess oil should be allowed to drain. The cryotube is sealed with cup.

Immediately after unfreezing (5 min in room temperature) the Sperm VD is placed in a pre-heated petri dish with PVP drops and sperm wash media covered with oil.

Sperm VD is quite convenient for rare sperm freezing and it gives the opportunity for direct sperm washing, selection and injection in the petri dish right after thawing.

7. Rare sperm freezing significance

Since the first attempts for freezing reproductive cells, a completely new horizon for fertility preservation has been discovered. The opportunity to cryopreserve, thaw and use gametes, embryos and reproductive tissue has given the unique chance to men and women to parent their biological child even when their reproductive functions have been lost.

Modern day society has invented and incorporated countless amenities in day to day life. Unfortunately, some of them have direct negative impact on health: polluted air as the result of factory overproduction, car exhaust gasses, overconsumption and large amount of waste contaminating water and agricultural lands, etc. Others tend to create bad habits: sedentary way of life and lack of physical activity, unhealthy and excessive eating, smoking, and drinking. Mental health, self-esteem and tension should also be considered when certain health issues arise. All of the above mentioned factors, including reproduction postpone and advanced paternal age, could disrupt fertility. Thus cryopreservation has great value in ART.

Cancer, the scourge of modern society, could affect reproduction and leave an indelible mark in one's life. Preserving fertility in cancer patients should be a priority as high as the treatment of the cancer itself – a guarantee for good quality of life after healing. Cancer by itself may affects fertility and alterations in spermatogenesis and low sperm quality could be registered even before treatment [69]. Rare sperm freezing of multiple samples for those patients is mandatory. The unique techniques developed for single sperm freezing and cryopreservation represent the chance for those patients to reproduce in future.

Rare sperm cryopreservation is of high value to patients with severe oligoasthenozoospermia, cryptozoospermia, retrograde ejaculation, and PESA or TESE procedures. All these conditions are associated with extremely low sperm count in the ejaculate and still creates difficulties for laboratory handling. Securing frozen samples through single sperm cryopreservation, inspires calm not only to the patient, but to the clinical and laboratory staff handling the gametes.

Rare sperm cryopreservation could be considered as opportunity beneficial to wildlife and endangered species preservation [70–72]. Breeding and assisted reproduction in animals has long-standing traditions and has been explored in husbandry for centuries. In times when hundreds of animal species are near extinction, rare sperm freezing is a key procedure for stock up of larger amount of samples for future breeding. Genetic diversity should be preserved and on time establishment of cryobanks conserving samples of different representatives of the species on Earth is of high importance [73].

8. Conclusion

Rare sperm freezing is a procedure with wide application and of high value in many aspects to ART, fertility preservation and endangered wildlife conservation. Freezing single sperm cells is still a challenge in modern laboratories. Incredible effort has been set and numerous devices and sperm freezing protocols have been investigated in order to establish the most reliable approach to rare sperm freezing. Despite the great achievements in the area, scientists continue the search for better results.

Conflict of interest

The authors declare no conflict of interest.

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"Many ideas grow better when transplanted into another mind than the one where they sprang up."

Oliver Wendell Holmes

Abbreviations

AR	assisted reproduction
ART	assisted reproductive technology
AZF	azoospermic factor
DMSO	dimethyl sulfoxide
DFI	DNA fragmentation index
ICSI	intracytoplasmic sperm injection
HA	hyaluronan
HA-Ph	hyaluronan -phenolic hydroxyl
HS	hemi-straw
OPS	Open pulled straw
PESA	percutaneous epididymal aspiration
PDMS	Polydimethylsiloxane
PROH	1, 2 propanediol
PVP	polyvinylpyrrolidone
ROS	reactive oxygen species
Sperm VD	Sperm vitrification device
TESE	testicular sperm extraction
SMF	severe male factor
SCO	Sertoli cell only syndrome
YCMs	Y-chromosome microdeletions
ZP	zona pellucida

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