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Cryopreservation of Human Spermatozoa: A New Frontier in Reproductive Medicine

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Abstract

Cryopreservation is a worldwide technique that makes it possible to preserve different living cells and tissues, including male and female gametes and embryos, in a structurally intact state using low temperature over time. Since the starting point of the cryopreservation era in 1776, until today, this was one of the most important steps in assisted reproductive techniques. Conventional slow freezing of spermatozoa is commonly used for cryopreservation of both ejaculated and surgically retrieved spermatozoa. The technique of the slow freezing is principally based on dehydration of cells which is performed through slow cooling combined with low concentrations of a cryoprotectant agent for achieving a balance. Besides of slow freezing, for more than a decade, many reports suggest the sperm vitrification technique as an alternative to slow freezing. Contrary to the slow freezing method, with vitrification, the effects of the cryoprotectants in spermatozoa are eliminated since this method is cryoprotectant-free. All of these interesting and promising protocols of vitrification, however, have not been implemented in the lab routine yet, and slow freezing remains the standard cryopreservation method in most laboratories worldwide.

Keywords: cryopreservation, vitrification, permeable and non-permeable cryoprotectants, human spermatozoa

1. Introduction

Cryopreservation, derived from the Greek word κρύος (krýos, "icy cold, chill, frost"), is a worldwide technique that makes it possible to preserve different living cells and tissues, including male and female gametes and embryos, in a structurally intact state using low temperature over time. It has become an indispensable part of most human-assisted reproductive technology (ART) programs around the world. Since the starting point of the cryopreservation era in 1776, Spallanzani [1] used snow to freeze spermatozoa and assess their motility after thawing. One century later, Mantegazza (reported by Curry [2]) observed that human sperm survived frozen at −17°C for more than 4 days. Since the late 1930–1940s [3, 4], scientists have effectively cryopreserved spermatozoa of several mammalian species, especially bovine and human. In 1947, glycerol was rediscovered as a cryoprotective agent allowing to freeze viable spermatozoa for longer periods. Bernstein and Petropavlovski [3] demonstrated the positive effect

of 1 mol/l glycerol on spermatozoa frozen to −21°C in the rabbit, guinea pig, bull, ram, stallion, and boar. After that in the late 1940s, the results of experiments based on the use of glycerol by Polge et al. [4] in the United Kingdom (1949) were published. The first piglet born from frozen–thawed porcine sperm in 1957 showed that the fertilizing potential could also be preserved and no major harm to the genetic apparatus was done [5].

Some cryopreservation methods developed in the 1950s are in use till now. However, the real success of cryopreservation was achieved in the 1970s with the introduction of dry ice and liquid nitrogen vapor. Following the report published in 1964, for the first time, human spermatozoa were successfully cryopreserved [6]. Definitely, this was one of the most important steps in assisted reproductive techniques, since the preservation of male sample in some conditions is the only opportunity to preserve fertility for some couples. Besides that, sperm cryopreservation is also considered as a rescue option for cases prior to radiotherapy and/or chemotherapy [7] in cancer patients, prior to any other medical procedure that may potentially lead to testicular failure or ejaculatory dysfunction as vasectomy [8], as well as in cases of traveling husbands serving in the military or absent partners. Even in pediatric oncologic cases, in which testicular tissue can be preserved, the cryopreservation of immature testicular tissue for later fertilization purposes seems also advisable. Generally, sperm cryopreservation when correctly performed allows long-term storage and usage when needed, which is one of the most essential parts in assisted reproductive techniques.

Complete cryopreservation as a technique consists three steps: the first one is a collection of the sample, the second is the freezing procedure, and the third is the storage part in liquid nitrogen [9]. For the male gamete cryopreservation, collecting a sample can be divided into collecting ejaculated spermatozoa, collecting epididymal spermatozoa, and collecting testicular spermatozoa. The first one, collecting ejaculated spermatozoa, should be always the first option when the patient is capable to provide a sample with adequate sperm viability; since intracytoplasmic sperm injection (ICSI) allows that sperm is directly injected inside of the oocyte, minimum requirement for semen parameters do not exist. On the other hand, the complete absence of sperm in the ejaculate following 2–3 days of abstinence on at least two occasions is the standard used to confirm the diagnosis of azoospermia; in this case, epididymal and testicular collecting of spermatozoa are usual procedures [9, 10]. Percutaneous epididymal sperm aspiration (PESA) does not require a surgical incision. A small needle is going directly into the head of the epididymis through the scrotal skin, and fluid is aspirated and examined for the presence of motile sperm. On the other side, microsurgical epididymal sperm aspiration (MESA) involves dissection of the epididymis under the operating microscope and incision of a single tubule. Fluid spills from the epididymal tubule and then is aspirated. Epididymal spermatozoa are mature and progressively motile, and epididymal aspirates are much cleaner and devoid of the cellular debris that is seen in testicular sperm preparations [10]. For the testicular sperm extraction (TESE) procedures, different techniques have been developed and compared. The microsurgical TESE seems to have the highest sperm retrieval rate and may limit damage to the testicular tissue. Spermatozoa can be retrieved from tubules that are dilated, and this can be visualized with an operating microscope. This technique needs microsurgical skills and general anesthesia is usually required. An open biopsy also allows the excision of a larger tissue mass, allowing access to a greater number of sperm available for freezing [10, 11]. Besides these freezing steps, the thawing procedure is an equivalently important step. Spermatozoa must be allowed to retrieve its normal biological activities. Generally speaking, at the present time, all the cryopreservation protocols use a thawing temperature of 37°C for 10 or 15 min [12].

2. The conventional "slow freezing" method

Conventional slow freezing of spermatozoa is commonly used for cryopreservation of both ejaculated and surgically retrieved spermatozoa. The technique of slow freezing is principally based on dehydration of cells [13] which is performed through slow cooling combined with low concentrations of a cryoprotectant agent for achieving a balance. Slow freezing of spermatozoa is commonly performed by stepwise manual or continuous programmed freezing of vials or straws, containing a mixture of cryoprotectants and spermatozoa, to subzero temperatures [14]. The manual method is performed by simultaneously decreasing the temperature of the semen while adding a cryoprotectant in a stepwise manner and after immersing the samples into liquid nitrogen [15]. Mahadevan et al. [16] reported that the optimal initial cooling rate of the specimen from room temperature to 5°C is 0.5–1°C/min. The sample is then frozen from 5°C to −80°C at a rate of 1–10°C/min and then submerged into liquid nitrogen at −196°C. Despite the fact many research presented successful sperm freezing with manual techniques, the reproducibility of this procedure pointed out some problems. For this reason, programmable freezers have been investigated [17]. These devices generally use a plate to hold the straws; these are cooled by liquid nitrogen held in a storage tank under the plate. Liquid nitrogen is poured into the tank, and the machine, once programmed, uses the software data logging to obtain cooling from 20°C to −80°C at a rate of 1.5°C/min and then at 6°C/min; at completion of the freezing, the straws are removed and stored in liquid nitrogen at −196°C. This takes about 40 min [17]. Software is simple to use and do not require continuous operator intervention and, besides that, have been used to increase the reproducibility of the freezing operations.

However, many research confirmed that the cryopreserved/thawed spermatozoa lose about 50–40% of their pre-freezing motility value, with considerable fluctuation among samples [18]. Although, after freezing, the regression of the motility is one of the first affected parameters, the mechanism of sperm impairment and its mechanical, physical, and/or chemical etiology is still the point of discussion. The ice equilibrium in conventional slow freezing is one of the main causes of mechanical cell injury, which result in the formation of intra- or extracellular ice crystal, along with the osmotic damage [19].

The cell further downgrades as well through consequent thawing and rewarming that affect their viability by possible excessive osmotic swelling. Chemical and physical damage are also caused affecting the sperm cell membranes as a result of expanded lipid peroxidation, due to the production of reactive oxygen species [20] which also lead to loss of sperm motility [21]. In order to prevent all the previously reported damages, during the time, different cryoprotectants were developed. Cryoprotectants are low-molecular-weight and highly permeable chemicals used to protect spermatozoa from freeze damage by ice crystallization. There are four main well-known cryoprotectants: glycerol, ethylene glycol, dimethyl sulfoxide, and 1,2-propanediol. Cryoprotectants decrease the freezing point of a sample, reduce the amount of salts and solutes present in the liquid phase of the sample, and decrease ice formation within the spermatozoa [22]. Usually, the cryoprotectants are added in an equal volume of semen in a dropwise manner, gently mixed at room temperature, and then placed at 37°C for 10–15 min to allow for proper equilibration between the cells and the medium [23]. Besides the permeable cryoprotectants, there are non-permeable agents, such as raffinose, sucrose, egg yolk citrate, albumin, polyethylene glycol, and polyvinylpyrrolidone, which are common additives that cannot pass through the plasma membrane but have antioxidant effects and are used to improve post-thaw sperm functional parameters such as post-thaw motility, viability, and reducing DNA damage [12]. Presently, slow freezing techniques have

been widely used in sperm cryopreservation with acceptable results for sperm vitality and motility after thawing [24]. However, for more than a decade, many reports suggest sperm vitrification technique as an alternative to slow freezing [25].

3. Vitrification as an alternative: new technique

In the past, the first successful vitrification of frog spermatozoa was done by Luyet and Hodapp [26] and fowl spermatozoa 4 years later by Shaffner [27]. In the early 1980s, Rall and Fahy [28] managed to successfully vitrify embryos using high concentrations of permeable cryoprotective agents and a relatively low speed of cooling and warming, and since then, the main approach to vitrifying spermatozoa has been considered the same as the methods used for other types of mammalian cells [29]. Vitrification as a method is based on the ultrarapid increase and decrease of temperatures with or without the use of non-permeable cryoprotectants. During the procedure, water is cooled to a glassy state through extreme increasing of viscosity without intracellular ice crystallization making this procedure less labor-intensive, faster, and presumably safer than traditional slow freezing protocols [30].

Contrary to the slow freezing method, the effects of the cryoprotectants in spermatozoa are eliminated since this method is cryoprotectant-free. Using this method, the sperm suspension is plunged directly into liquid nitrogen, and the sperm cells are cooled in an ultrarapid manner, known as kinetic vitrification [25]. What is more, cryoprotectant-free technique avoids the use of the classic toxic cryoprotective agents (CPA) that may have lethal effects of osmotic shock and prevent lethal intracellular ice formation and the harmful effects of high salt concentrations during freezing and thawing [25]. The first described cryoprotectant-free vitrification by Nawroth et al. [31] suggested that spermatozoa were located onto copper loops or into standard 0.25 ml insemination straws and plunged directly into liquid nitrogen. During the years of improving technique, different devices have been tested [30, 32, 33]. Isachenko et al. [34] compared the vapor phase and liquid phase for sperm cryopreservation, using the cryoloop, droplet, and open straw methods. Cryoloops with a film of spermatozoa suspension were cooled for 3 min in liquid nitrogen vapor at −160°C and then placed into pre-cooled cryovials (CryoTubesTM, 4.5 ml volume, 92 mm length; Nunc GmbH & Co. KG, Wiesbaden, Germany) and were stored in liquid nitrogen until the time of warming. The second developed method at that time was vitrification in liquid nitrogen vapor using droplets where sperm suspension was located onto aluminum foil previously cooled in liquid nitrogen vapor to −160°C. During cooling, the droplet of sperm suspension adopted a spherical form, which was placed into pre-cooled cryovials and stored in liquid nitrogen. With open straw method, sperm suspension was drawn inside the end of open-pulled straws (0.25 ml) (Medical Technology GmbH, Altdorf, Germany) by capillary action [32]. Straws were placed inside other sterile 90 mm straws which were prepared from the standard 0.5 ml insemination straws (Medical Technology GmbH, Altdorf, Germany) and then hermetically closed using a handheld sealer and then plunged into liquid nitrogen. During this procedure, it was described that there was no contact between the wall of the 90 mm straw and the suspension of spermatozoa inside the open-pulled straws, due to the presence of a meniscus in the suspension [34]. At the end, the results report that CPA-free cryopreservation of spermatozoa could occur in a wide range of cooling rates, but the major disadvantage of cryoprotectant-free vitrification was that only small volumes of spermatozoa (≤40 μl) could be vitrified in these systems [32].

What is more, in the aim to improve the cryoprotectant-free vitrification, Schulz et al. [35] added some carbohydrate supplements (glucose, sucrose, and trehalose)

to the sperm suspension and obtained that sucrose has increased spermatozoa motility and viability after thawing. Later on, Isachenko et al. [36] investigates the ability of sucrose to protect sperm motility, viability, mitochondrial membrane potential integrity, spontaneous capacitation, and acrosome reaction. Spermatozoa were cryoprotectant-free vitrified using three different media: human tubal fluid medium (control), human tubal fluid medium with 1% human serum albumin, and fluid medium with 1% human serum albumin and 0.25 M sucrose [36].

Obtained results in this research [36] indicate that the number of progressively motile spermatozoa was significantly higher in the sucrose-supplemented medium group (57%) than with controls (19%). The combination of fluid medium with 1% human serum albumin and sucrose (65%) has a stronger cryoprotective effect on the integrity of mitochondrial membrane potential than with human tubal fluid medium with 1% human serum albumin alone (33%). It was concluded that cryoprotectant-free vitrification of human spermatozoa with non-permeable cryoprotectants such as human serum albumin and sucrose can effectively cryopreserve the cells without significant loss of important physiological parameters. On the other hand, Chen et al. [37] reported that normozoospermic patient samples vitrified by the cryoprotectant-free method with or without the addition of sucrose did not show a significant difference in the sperm recovery rate and motility rate.

During the years, many researchers got their attention into the vitrification of oocytes, embryos, larger cells, and even tissues [38–40]. It was established, at that time, that vitrification for oocyte and embryo could not be achieved without combinations of high concentrations of both permeable and non-permeable cryoprotectants in order to reach stable vitrification and allow using a relatively low rate of cooling and warming. Mainly dimethyl sulfoxide, propanediol, or ethylene glycol were used for ooctyte and embryo vitrification [41–43]. However, these methods also have their limitations, and especially human spermatozoa are intolerant to the high concentrations of cryoprotectants conventionally used in vitrification [44, 45]. Suggested reasons for successful vitrification in the absence of cryoprotectants for spermatozoa are the size of the cells and their relative concentration of soluble macromolecules. The shape and size of the sperm head could define the cryo-sensitivity of the cell. Comparative studies [46] have shown a negative correlation between the size of the sperm head and cryo-stability. Oocyte and spermatozoa naturally contain high concentrations of proteins, which help in vitrification. Thus, a higher cryoprotectant concentration is needed for extracellular vitrification than for intracellular vitrification [25]. It can be assumed that the amount of osmotically inactive water is also higher in spermatozoa and is combined with several macromolecular structures such as DNA, histones, etc. [47]. Extensive classification of intracellular compounds may also contribute to the successful survival of spermatozoa [48].

In spite of this, vitrification of spermatozoa is still a rather unexplored methodology, with limited studies showing its efficacy in male gametes [24, 25, 30–32]. In the last two decades, different sperm vitrification protocols have been published, most of them developed by Isachenko [30–32, 34, 36] where not only different combinations of cryoprotection but also different devices have been tested as well. These interesting and promising protocols, however, have not been implemented in the IVF lab routine yet, and slow freezing remains the standard cryopreservation method in most laboratories worldwide [49].

4. Our experience

Since there is no optimal accepted algorithm for the vitrification procedure and no evidence has been established regarding the possibility of achieving successful

human spermatozoa vitrification without cryoprotectant, the differences between vitrification of human sperm with and without two non-permeable cryoprotectants (NPC), sucrose (SUC), and trehalose (TRE) were investigated. For that, five human semen samples diagnosed as normal (*normozoospermia)* were obtained and analyzed. All samples were prepared through 80% one-step density gradient and then were diluted to a defined sperm concentration of 40×10^6 sperms per ml. following the guidelines of the World Health Organization criteria [13]. Motility and vitality of the sperm were recorded in each sample. Each sample was then divided into three parts. Each part of the samples underwent vitrification using three different methods: (i) 150 µl cryostraw filled with the sample was directly plunged into liquid nitrogen, (ii) small drops of the samples were dripped into liquid nitrogen, and (iii) microcapillaries were plunged directly into liquid nitrogen. Each part was vitrified differently using sucrose and trehalose or without a cryoprotectant. Sucrose and trehalose were diluted in media, 500 mM, after merging it with the sperm solution concentrations till they have reached 250 mM of concentration with 20×10^6 sperms per ml. After a minimum of 24 hours of cryopreservation, samples were thawed in warm water at 37°C for 20 seconds. Then, the samples were washed in 1 ml media (SpermActive®; Gynemed) and incubated at 37°C. Motility and vitality were directly assessed, 30 and 120 min after thawing. It was found that sperms' overall survival rates ranged from 1.2 to 37.5%. Mean survival rates for each method were as follows: method (i), 15.5 ± 5.3% for sucrose, 16.6 ± 5.2% for trehalose, and 19.8 ± 7.8% without cryoprotectant; method (ii), 47.8 ± 16.6% for sucrose, 53.8 ± 13.4% for trehalose, and 40.4 ± 7.5 without cryoprotectant; and method (iii), 10.3 ± 2.9% for sucrose, 8.2 ± 3.9% for trehalose, and 8.2 ± 3.3% without cryoprotectant (**Table 1**). Statistical analyses revealed significant differences only between method (ii) and method (iii) (**Table 2**), with a tendency for better survival rates in method (ii) under all three conditions, especially with trehalose (mean survival rate of 53.8 ± 13.4%; **Table 1**). However, these differences are not representative because of the low survival and motility rate after thawing for method (iii), which might be affected by the heat sealing process (sealing small volume of the sample). No significant differences were observed regarding the addition of sucrose and trehalose or vitrification without a cryoprotectant in terms of overall survival rates (suc. vs. no cryoprotectant n = 5, p > 0.4; tre. vs. no cryoprotectant n = 5, p > 0.3; **Table 2**). Similarly, no significant differences were assessed between the use of sucrose and trehalose (n = 5, p > 0.7). Mean motility recovery, measured after 120 min of thawing, was 13.7 ± 5.9% for sucrose, 12.8 ± 4.2% for trehalose, and 10.1 ± 4.3% without cryoprotectant, and

	N	Mean Survival
Method (i) + sucrose	5	15.6 ± 11.7%
Method (i) + trehalose	5	16.6 ± 11.6%
Method (i) without NPC	5	19.8 ± 17.5%
Method (ii) + sucrose	5	47.8 ± 16.6%
Method (ii) + trehalose	5	53.8 ± 13.4%
Method (ii) without NPC	5	40.4 ± 7.5%
Method (iii) + sucrose	5	10.3 ± 2.9%
Method (iii) + trehalose	5	8.2 ± 3.9%
Method (iii) without NPC	5	8.2 ± 3.3%

Table 1.
Mean survival rates for all three methods and conditions.

	Sucrose	Trehalose	without NPC
(i) vs. (ii)	P=0.207	P=0.217	P=0.131
(i) vs. (iii)	P=0.38	P=0.171	P=0.194
(ii) vs. (iii)	P=0.007	P=0.002	P=0.002

Table 2.
Comparison of the different methods.

between these three groups, there was not a significant difference. However, interestingly, a tendency of improved motility was observed during post-thawing incubation specifically in addition of cryoprotectants (**Figure 1**). Therefore, the choice of the vitrification method and conditions seems to influence survival rates, motility, and vitality of the sperm, but the significant difference in sperm recovery after vitrification with non-permeable cryoprotectants was not found; the reason
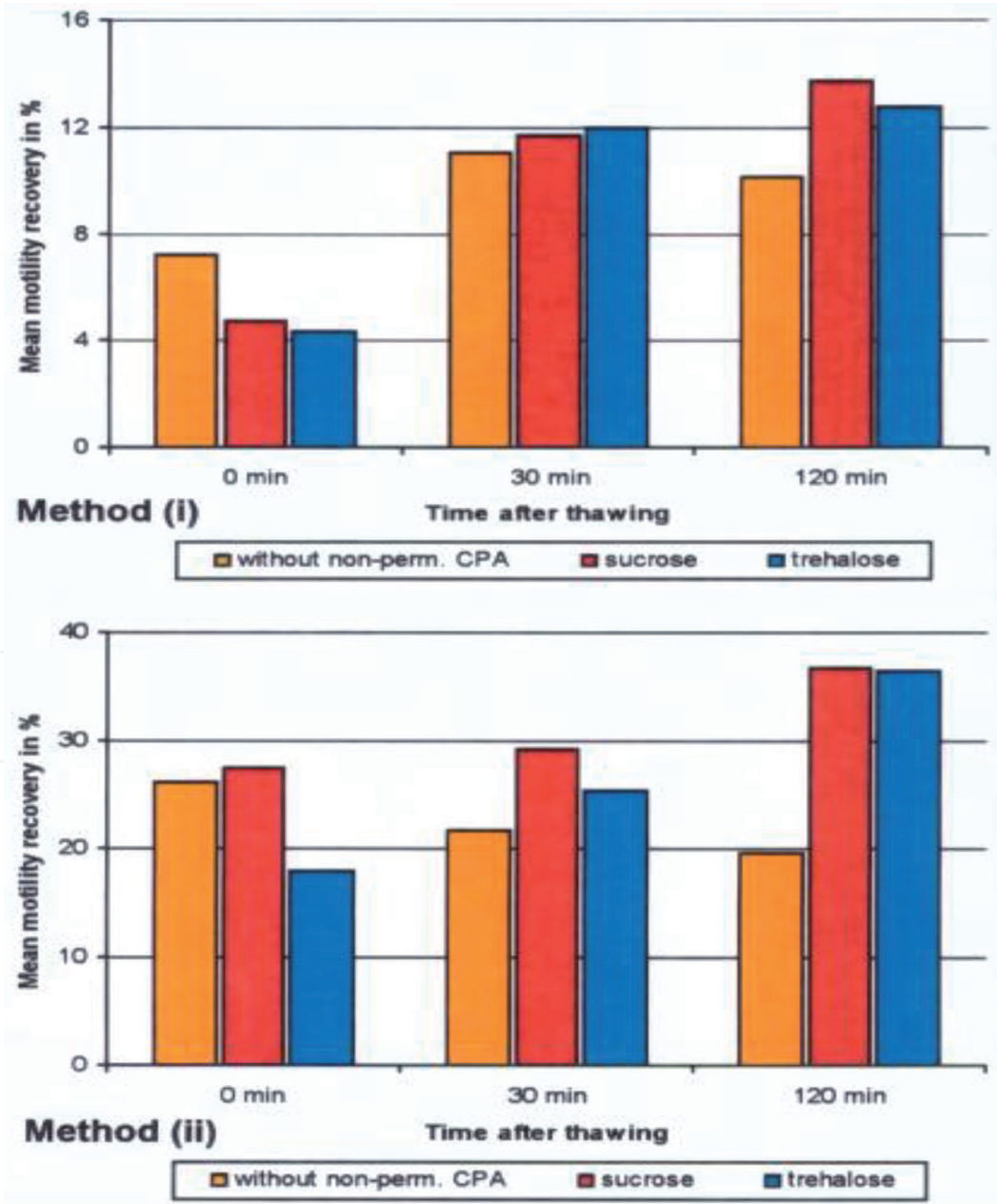

Figure 1.
Recovery of motility by post-thawing incubation.

that influenced the obtained result might be the small sample size. On the other hand, the improvement of the methodology of sperm vitrification could yield in positive additional effects of non-permeable cryoprotectants. Under these conditions, obtained data might be encouraging for further studies, to extend on a greater number of normal sperm samples as well as to those patients with reduced semen quality and fertility problems. Moreover, the increase in post-thaw sperm motility is an important aspect in the use of all assisted reproduction techniques and should be also confirmed by further studies.

5. Conclusion

Spermatozoa cryopreservation for males is the standard fertility preservation care in patients undertaking gonadotoxic treatments, such as chemotherapy/ radiotherapy. The conventional cryopreservation, the slow freezing technique, is standardized and commonly used. However, functional sperm parameters including motility after the thawing are still challenging. New methods that preserve spermatozoa are promising, even though they still need validation before being routinely used in an assisted reproduction program, including the essential use of new cryoprotectants and new antioxidants to improve sperm quality after thawing. Moreover, spermatozoa lyophilization is another method that is still under investigation. However, as the spermatozoa are immotile, lyophilized sperm can only be used in ICSI. Hence, future research needs not only to investigate the optimization and safety of methods but also for the health of the offspring.

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