

# HUMAN SPERM CRYOPRESERVATION

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## ABSTRACT

As the demand for assisted reproductive techniques in humans increases, so does the demand for the oocytes and sperm that are essential for these techniques. Human sperm banks play a key role in assisted human reproduction, as a reservoir of sperm from the semen of donors and as an option for genetic preservation for some patients. There are different techniques that can be used to store human sperm. This paper will provide an overview of the available techniques of human sperm preservation.

**Keywords:** Fertility preservation, vitrification, freezing-thawing, cryopreservation, sperm banks.

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## INTRODUCTION

Approximately 10-15% of couples worldwide who are trying to have children experience some form of infertility.<sup>1</sup> For these couples, problems with the sperm (entirely or in combination with female factors) account for 50% of the infertility problems.<sup>2</sup> These infertility issues are not only important in developed countries, but also in developing countries where the demand for assisted reproductive techniques (ART) as a treatment for infertility is increasing.<sup>3</sup> In addition, the demand for artificial insemination (AI) or ART is also increasing for non-traditional families, such as single women or homosexual couples.<sup>4</sup> To date, more than 5 million children worldwide have been born through ART (data that exclude those coming from AI),<sup>5</sup> and the number of children born using ART represented 1.73% of all children born in the year 2002 in the European countries that participated in the European registers.<sup>6</sup>

The limited lifespan of fresh sperm requires significant coordination in order to retrieve both sperm and oocytes at the same time,<sup>7</sup> which may be inconvenient or impossible in some cases. For this reason, research involved with extending the lifespan of the sperm, by cooling the cells, has

been pursued. Generally speaking, the lower the temperature at which the sperm is stored, the longer the cell viability is maintained.<sup>8</sup> However, the fertilising ability of fresh sperm declines sharply after very few days of storage at 5°C.<sup>9</sup>

## LONG-TERM STORAGE OF SPERM: AND SO IT BEGAN

Mantegazza observed in 1886 that human sperm survived cooling to -17°C for more than 4 days (reported by Curry<sup>10</sup>). However, 4 days does not extend the lifespan of the sperm sufficiently long for most purposes, and research was conducted to develop methods (extenders and protocols) for longer storage periods. Sixty years later, researchers discovered that glycerol was an effective cryoprotective agent (CPA) for freezing sperm.<sup>11</sup> Since then, glycerol has been the primary CPA used for freezing sperm from most animal species, including humans.<sup>12</sup> The discovery that glycerol was an effective CPA quickly resulted in the development of cryopreservation techniques that maintained not only sperm motility after freezing and thawing, but also fertilising ability; in 1953, Bunge and Sherman reported the first pregnancy resulting from cryopreserved human sperm, which had been frozen using glycerol and

stored on dry ice. However, it took another 20 years of research before the first commercial sperm banks were created in the USA (reviewed by Frankel<sup>13</sup>).

## USES OF CRYOPRESERVED SPERM

Cryopreservation permits long-term storage of semen and, when cryopreserved correctly, the sperm maintains a state of metabolic arrest that prevents cellular ageing and retains the viability and fertilising ability of the sperm for an essentially unlimited period. In addition, cryopreservation can inhibit the transmission of several infectious diseases that can be transmitted through semen (such as HIV), leading to the requirement that semen from donors be cryopreserved. By using only frozen semen, possible disease carriers can be detected during a quarantine period,<sup>14</sup> and the risk of transmitting infectious agents is almost non-existent as long as the safety regulations are followed.<sup>15</sup>

Sperm cryobanks are currently used for several purposes.<sup>16</sup> Donor sperm can be used by women with or without a male partner, and cryobanking sperm for future use can be used for male patients undergoing procedures that would impair or curtail fertility (vasectomy, treatments with cytotoxic agents, or radiotherapy) or those who are exposed to sperm-damaging conditions in their professional career but would like to become fathers in the future. Cryobanks can also collect and store gametes needed in infertility cases in which the male partner cannot provide sufficient or suitable sperm for use in ART, or cases in which it is not possible to collect fresh semen on the day of the ART procedure. Finally, cryobanks are useful for storing samples from disease carriers (such as HIV) and minimising the risk of disease transmission to the female partner by storing and using only those samples with an undetectable infectious agent.

## SPERM CRYOPRESERVATION

As previously stated, long-term storage of cells can be achieved if metabolism is arrested, and this can be achieved if the cells are stored at  $-196^{\circ}\text{C}$  – the temperature of liquid nitrogen. At  $-196^{\circ}\text{C}$  there is essentially no detectable biochemical activity<sup>17</sup> because there is not sufficient thermal energy for chemical reactions to occur and there is no liquid water, which is essential for metabolic processes.<sup>18</sup> However, living tissues and cells can be destroyed during the freezing and warming processes unless

certain procedures are used. CPAs are added to the diluents for cryopreserving the sperm. They protect the sperm during the cryopreservation process and, as a result, the sperm cryosurvival rate is higher in their presence than in their absence.<sup>17</sup> There are two types of CPA differentiated by their ability to traverse the plasma membrane: permeating and non-permeating CPAs.

The freezing point of water is close to  $0^{\circ}\text{C}$  in the presence of nucleating particles, but in their absence it can be reduced to  $-42^{\circ}\text{C}$ .<sup>17</sup> During the cryopreservation protocols, the solutions cool below their normal freezing points without changing their state from liquid to solid (ice). This phenomenon is known as supercooling and it is due to the need for nucleation to occur (the process by which a minimum crystal is formed) before an ice crystal can begin to grow. Thus, when the ice nucleus begins to grow, the solutes are excluded from the ice crystals and they concentrate in the unfrozen (liquid) fraction, and this lowers the freezing point of the remaining solution. Ice crystals continue growing until the solution at the interface has a freezing point equal to the temperature of the interface. Nucleation and ice crystal formation can be avoided if the temperature is reduced very fast in a process known as vitrification.<sup>17</sup> Currently, two techniques are effective for preserving cells: slow (equilibrium) freezing and vitrification.

## SLOW-FREEZING PROTOCOLS

In these protocols, extracellular water solidifies into ice crystals and creates a two-phase state: ice crystals of pure water and an unfrozen fraction containing liquid water and all the salts, sugars, CPA, and cells of the original sample (for reviews see Chian<sup>17</sup> and Hammerstedt et al.<sup>19</sup>). The change in temperature alters the physical status of the sperm membrane as membrane lipids undergo a phase transition from the fluid to the gel state, which can damage the membrane.<sup>19</sup> In addition, the loss of water, in the form of ice, increases the osmolality of the solution in the unfrozen water fraction, which can also damage the cells.<sup>19</sup> Since sperm lack the ability to adapt to low temperatures (for a review see Parks<sup>8</sup>) we must provide the sperm with CPAs (such as glycerol and egg yolk) in order to help them survive these hypothermic conditions.

The beneficial components in egg yolk are the low-density lipoproteins that protect the sperm membrane during cryopreservation.<sup>20</sup> Glycerol acts as a permeating CPA and exerts its effects both

intracellularly and extracellularly. Extracellularly, glycerol lowers the salt concentration of the extracellular fluid and increases the percentage of unfrozen water fraction at any given temperature, minimising the osmotic effect.<sup>21</sup> Both egg yolk and glycerol are commonly added to the human sperm at room temperature, and the samples are equilibrated with the CPAs for several minutes (commonly at 35°C for 30 minutes<sup>16</sup>), after which the sperm are loaded into cryovials or straws. There is no consensus regarding an optimal freezing diluent or freezing protocol for human sperm. Therefore, practitioners can choose between different protocols depending on the equipment available to them and on the type of sample to be cryopreserved (for a review see Di Santo et al.<sup>22</sup>).

Regardless of the protocol chosen, some precautions should be taken in order to achieve optimal results. Firstly, although glycerol helps sperm survive the freezing process it also can have deleterious effects and, at high concentrations, can be cytotoxic.<sup>23</sup> However, this toxic effect is observed at concentrations higher than those used for freezing sperm (around 6% v/v). Secondly, glycerol exerts direct osmotic effects on cells. Glycerol crosses the sperm plasma membrane, but at a slower rate than water. Therefore, when glycerol is added or removed, the cells experience volume changes (shrinking and expansion, respectively) that can damage the cells if the volume changes surpass the osmotic tolerance limits of the sperm (for a review see Gao et al.<sup>14</sup>). To avoid this damage when adding the CPA, the freezing diluent is added to the sperm in several steps.<sup>14</sup> After adding CPA, the sperm are equilibrated with the freezing diluent for several minutes prior to actually freezing the sperm. During this period, the glycerol traverses the membrane, equilibrium is reached, and apparently protective membrane rearrangements occur, although this remains controversial.<sup>14</sup>

The temperature must also be carefully reduced, even in the presence of CPAs. For the avoidance of iatrogenic damage, the temperatures of the sperm, the diluents, and the instruments used to manipulate the samples must be similar. In addition, sperm can be damaged by decreasing the temperature from body temperature to near 0°C too quickly (for a review on the effects of hypothermia on sperm see Parks<sup>8</sup>). This damage, known as cold shock, correlates directly with temperature differential and cooling rate (for an extensive review see Watson<sup>24</sup>). The resistance

of the sperm to this damage depends on the characteristics of the sperm membrane, lipid type, and cholesterol level, which are specific to different species. Therefore, resistance to cold shock is higher for sperm with ratios of polyunsaturated to saturated fatty acids in the membrane that are lower ( $\approx 1$ ), and cholesterol to phospholipid molar ratios that are higher ( $\approx 1$ ).<sup>24</sup> For human sperm, both molar ratios are close to 1, and for this reason human sperm are relatively cold-shock resistant. The implication for this is that the cooling/freezing rate for human sperm can be relatively fast, much faster than is used for sperm from many other species.

Freezing is performed in liquid nitrogen vapours. The cooling rates also have to be carefully chosen in order to avoid damaging the sperm. If sperm are cooled slowly then they will dehydrate and intracellular ice formation will be avoided, but they will be damaged by the high hyper-osmotic conditions created while the ice is growing. Fast cooling rates are also damaging because the water cannot leave the cells fast enough and it will supercool, which increases the probability of intracellular ice formation. The cooling rate also depends on the concentration of CPA.<sup>25</sup>

After freezing, the sperm are stored in liquid nitrogen where they remain essentially inert as long as they are kept at -196°C. However, before they can be used for AI or ART, they must go through a reverse process. To avoid cellular damage during thawing (either from recrystallisation of small crystals or from osmotic unbalance), the warming rate needs to be carefully chosen. The warming rate depends on the freezing rate, on the type of container used to load the sperm, and on the concentration of CPA.<sup>19</sup> As with freezing human sperm, several protocols for thawing human sperm have been described (usually the samples are thawed at either 37°C or at room temperature; for a review see Di Santo et al.<sup>22</sup>).

In addition, the freezing diluent is usually removed before using the sperm for ART. This step is of special concern because the sperm are now loaded with glycerol, and diluting the sperm in a diluent containing no glycerol will induce osmotic stress to the cells, which can swell to the point of being damaged. To alleviate this problem, the sperm should be diluted gradually using several steps with sufficient time for the sperm to equilibrate to each new osmotic condition.<sup>14</sup>

Slow-freezing protocols are still inefficient considering sperm cryosurvival and the functionality of the sperm surviving the process, as will be discussed later. In some patients, the percentage of sperm surviving the process can be too low to perform AI and these samples should be combined with other ART (such as *in vitro* fertilisation [IVF] or intracytoplasmic sperm injection [ICSI]). Nevertheless, slow-freezing protocols remain the most commonly used for the long-term storage of sperm.

## SPERM VITRIFICATION

During vitrification, water solidifies as an amorphous glass-like structure, and not as ice.<sup>17</sup> Vitrification is achieved by cooling the solution at very high rates, and the process has some advantages over slow-freezing protocols. Thus, vitrification should cause little damage to the cells, minimal equipment is required, and little time is devoted to the process (the cells are plunged directly into liquid nitrogen). Indeed, if extremely high cooling rates are used, isotonic saline can be vitrified<sup>25</sup> and no CPAs are necessary, eliminating all the osmotic challenges that adding and removing CPAs involve.

Although it seems easy to implement, the reality is that effectively vitrifying sperm has been difficult. This is due to the difficulty in cooling the samples sufficiently fast enough for vitrification to occur. In order to vitrify many cell types (such as oocytes or embryos), high concentrations of CPAs are used (as high as 30–50%), and sample volumes are small.<sup>25</sup> However, sperm do not tolerate these high CPA concentrations and, for this reason, vitrification has not been considered a real possibility for conserving sperm. In 2002, the successful vitrification of human spermatozoa was reported by the direct plunging of small sperm volumes (20  $\mu$ L) into liquid nitrogen without permeating CPAs.<sup>26</sup> Researchers demonstrated that the sperm maintained motility after warming and exhibited fertilising capacity. There are some problems with this type of conservation, including the risk of infectious agent transmission (the samples are in direct contact with the liquid nitrogen), difficulty in labelling the samples, and the small volumes used (insufficient for AI and for some ART). Thus, other investigations were conducted to resolve these issues (for an extensive review see Isachenko et al.<sup>27</sup>). In addition, special attention must be paid during the warming process because the warming

velocity plays a crucial role in the sperm cryosurvival of the vitrification–devitrification process.<sup>27</sup>

Vitrification is less popular than slow-freezing procedures nowadays and research is still being conducted to optimise the technique. However, the sperm obtained with this method present similar or higher quality than the sperm frozen with slow-freezing protocols. In addition, vitrified sperm are also fertile by means of IVF/ICSI and intrauterine insemination, although reports are still scarce. Nevertheless, a few centres are using vitrification for long-term sperm storage with successful results,<sup>28</sup> and this number will probably increase in the following years because the technique is quite simple to perform.

## OTHER TECHNIQUES FOR LONG-TERM STORAGE OF SPERM: FREEZE-DRIED SPERM

So far we have described techniques that require liquid nitrogen for storing the sperm. However, a continuous liquid nitrogen supply is necessary to maintain the samples, which makes this type of conservation costly. Therefore, other techniques that do not require liquid nitrogen are being developed for long-term sperm storage.<sup>29</sup> Lyophilising or freeze-drying sperm allows the sperm to be stored at suprazero temperatures. In this case, the samples are dehydrated by sublimation (for a review see Gil et al.<sup>30</sup>) and then stored at either 4°C or room temperature. Although the first attempts to lyophilise human sperm were made in the early 1950s,<sup>31</sup> no protocol to date has succeeded in maintaining motile or viable sperm. Nevertheless, the DNA of lyophilised sperm remains intact even though the other sperm structures are irreversibly damaged during the lyophilisation process,<sup>32</sup> and freeze-dried sperm used for ICSI have resulted in viable offspring for several animal species.<sup>30</sup> However, reports on the fertilising ability of lyophilised human sperm are lacking. Currently, lyophilisation must be considered as an experimental technique that should not be offered to patients because there is no information about the fertilising ability of this type of sperm in humans. Thus, this technique is still in the early stages but may become more promising as research continues to improve both the composition of the diluents used for lyophilisation and procedures for dehydration alternative to lyophilisation (for a review see Loi et al.<sup>29</sup>).

## ARE THESE TECHNIQUES SAFE?

All of the techniques described above damage sperm more or less severely, since nearly half of the sperm are irreversibly damaged during cryopreservation.<sup>33</sup> On the other hand, the sperm from some individuals are not damaged as severely by the freezing process.<sup>12</sup> However, only 10% of human sperm donors provide sperm that can be effectively cryopreserved.<sup>34</sup> This would be a potential problem for preserving sperm samples from patients in fertility preservation programmes, whose sperm will not be suitable for the simple reproductive techniques (such as AI). In addition, the fertility of cryopreserved sperm is lower than that of fresh semen when used for AI,<sup>35</sup> which is similar to the fertility of fresh and frozen animal sperm.<sup>12</sup> This reduced fertility is due to sperm membrane damage that occurs during cryopreservation, which compromises membrane structure and function (for a review see Parks<sup>8</sup>). However, the fertility of frozen-thawed semen improves if high-quality insemination doses are

used.<sup>35</sup> Therefore, whenever possible, high-quality sperm should be used for AI. However, when high-quality semen samples are not available, a number of ART have been developed and fertility can be achieved with cryopreserved sperm using IVF or ICSI.<sup>35</sup> Finally, the technique of cryopreservation is safe because there is no evidence of additional risks for birth defects or chromosomal abnormalities after using cryopreserved sperm.<sup>5</sup>

## CONCLUSION

Several techniques are available for the long-term storage of human sperm. Among them, slow-freezing cryopreservation protocols are used most often. Cryopreserved sperm maintain their fertilising ability for an extended period, although the fertility of samples tends to be reduced compared with fresh semen, but only if AI is used. Therefore, the advantages of cryopreserved sperm surpass its disadvantages and cryopreservation is an important tool for the preservation of male gametes.

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