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Human Sperm Freezing: Mini Update

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Abstract

Sperm freezing is widely used in ART clinics around the globe. Very little has actually changed with respect to cryopreservation protocols and methodology of freezing over the last 50 years. The aim of this paper is to briefly review the basic principles that underlie freezing and ice crystal formation and also provide a brief overview of newer sperm freezing techniques like sperm vitrification and freeze drying of sperm.

Keywords

Sperm Freezing, Principles of Freezing, Freezing Injury, Ice Nucleation, Cryoprotectants, Sperm Vitrification and Freeze Drying Sperm

1. Introduction

Cryobiology has colossal applications in the field of reproductive medicine. Freezing of sperm, oocytes and embryos is now routinely done in most ART (Assisted reproduction techniques) clinics around the globe [1]. Despite the rapid growth of this field, much work needs to be done in refining and defining different freezing protocols for both the male and female gametes and/or embryos. The core objective of cryopreserving gametes is to achieve good cell functionality and viability on thaw for clinical use [2]. Outcomes of freezing however depend on numerous factors some of which are modifiable, while some are not. Cell survival and/or functionality post thaw depends on the choice of freezing methodology used, the choice of cyroprotective agents utilized in the freezing protocol and to a certain extent on operator technical skill; at least for a few cell types [3]. The aforementioned factors are to a certain extent modifiable in nature.

Non-modifiable factors that affect freezing outcomes, depend on the inherent susceptibility of the cell/cells in question to freezing injury which to a certain

extent also depends on the cell type and/or individual's disease/health state [3]. An example would be that in men whose semen samples are normozoospermic in nature, post thaw sperm survival is usually excellent, even up to 5 times after refreezing and thawing the same sample [4] [5]. On the other hand, in men diagnosed with oligozoospermia, post thaw recovery is significantly poor even after one single freeze thaw cycle [4] [5].

Much of the research work on the effect of low temperature on different organisms was done by Luyet [6]. It is now well accepted and well known that some living organisms can virtually bring their body metabolism to a stop when subjected to extremely low temperatures. In essence, in these organisms ageing stops, cryobiologists frequently use the term "suspended animation". If life, by definition is considered a series of chemical reactions/events ultimately leading to death, then stopping these chemical reaction/events would bring biological ageing to a halt. Time would stop for these organisms [7]. These organisms would be projected forward into time much akin to the like's of using a *time machine*. Thus, the field of cryobiology holds tremendous potential in manipulating the time dimension of either a cell and/or organ and/or an individual [7].

A few bacteria, plant cells and metazoans have the ability to survive vitrification (ultra-rapid cooling) when exposed to liquid gases. This phenomenon prevents ice crystal formation inside the cell by allowing the water to directly transition to a *glassy state*, thereby preventing cellular injury. Vitrification requires ultra rapid cooling (2000˚ Celsius min^{-1}), only then can an extreme increase in viscosity occur thereby preventing ice crystal formation and also in parallel facilitating a direct transition of the cell to an innocuous glass state [8].

The lesser the water content of a cell, the better is its ability to withstand vitrification [8]. The faster the drop in temperature the better the outcomes of vitrification in general. Luyet and Hodapp had both vitrified an appreciable number of frog sperm, in 1938, with the help of hypertonic sucrose solution [9]. Shettles in 1940, first vitrified a few percent of human sperm by using a capillary glass inserted into liquid air followed by rapid warming [10]. However subsequent efforts made at vitrifying sperm were not successful, due to the inadequate rate or speed of cooling.

2. Principles in Freezing

An important distinction should be made between ultra rapid freezing (vitrification) and slow cooling/freezing. But, first an effort should be made to understand the basic principles of freezing. Freezing in general terms; is not just a simple physical phenomenon of water turning into ice. When a biological sample is frozen, two phenomenon occur.

1) There is a distinct slow down in the rate of metabolic reactions, and the extent to which these reactions would slow, depends on the reaction itself and also on other interdependent processes [11]. Interestingly, other chemical reactions

can still occur at these low temperatures.

2) A thermodynamic phase change occurs, the best example being the change of water in to ice [11].

Penultimately though, the change of physical state from water to ice is the single most important phenomenon to discuss. Ice formation first takes place outside the cell with the formation of an ice nucleus (localized formation of a distinct thermodynamic phase). A nucleation event occurs on "hydrophilic sites" (water friendly sites) that are present on the surface of the liquid. The presence of these inclusion sites allows crystal development to take place. This form of nucleation is also called "heterogeneous nucleation" and is the most common type of nucleation event seen in different biological systems [12].

This nucleus formed, will then reach a critical size, which will then determine whether there is an equal probability of the ice crystal either disappearing or growing [11] [12]. The critical size of the ice nucleus depends on the temperature. At higher temperatures or temperatures closer to the freezing point of water, the critical size of the ice nucleus is so large, that there is lesser chance for the random aggregation of water molecules to form ice [11] [12] [13]. The critical size could be large enough that even at the temperatures lesser than the freezing point of liquid water (0 degree Celsius), ice crystal formation may not occur, thus the resulting water becomes super cooled without freezing. However, as temperatures fall further fall, this critical size also decreases and there is better probability for ice formation.

Once the temperature approaches −40 degree Celsius, liquid water would spontaneously freeze into ice, and this temperature is frequently accepted as the "homogenous nucleation" temperature for liquid water. This temperature is also the limit of super cooling of liquid water and at this temperature even finely dispersed water droplets spontaneously freeze to form ice [14]. The presence of solutes in liquid water can frequently lower both the temperature of homogenous and heterogeneous nucleation. For all practical purposes, as far as biological systems are concerned, homogenous nucleation does not occur, due to the presence of complex solutes, membranes and other dissolved substances [11] [12] [13] [14] [15]. For homogenous nucleation to occur, water has to be ultra pure and should contain no known ice nucleators.

Ice crystal growth: Once an ice crystal forms, it will continue to grow in size. Ice has a very poor ability to dissolve solutes and thus when an ice crystal grows, the solutes are excluded in the remaining unfrozen water fraction [16]. This causes the solute concentration to rise in the unfrozen water fraction. An example of this effect would be freezing isotonic saline, the NaCl concentration rises over 32 fold above baseline concentration at −32 degree Celsius [16]. The initial composition of the solution has little effect on ice crystal growth, but can definitely influence the temperature at which crystallization can occur.

The growth of ice crystals depends more on the rate of cooling. When the rate of cooling is extremely fast, small ice crystals form and they have insufficient

time to grow, thereby allowing the remaining un-crystallized water to nucleate at a lower temperature. However, when cooling is slow there is sufficiently adequate time to allow crystal growth in terms of size thereby resulting in the formation of larger crystals [16] [17] [18]. Ice crystal growth does not however freeze all the water in a system. Water has numerous complex functions, and tightly bound water that constitutes 5% - 10% of the most biological tissues would never freeze [18].

Freezing injury: Living cells have the ability to supercool to about −40 degree Celsius without intracellular ice crystal formation. Cells in general do not contain ice nucleators, and ice formation most often begins in the extracellular compartment specially during slow cooling [19]. The extracellular ice, removes water from the solution thereby concentrating solutes and dehydrating the cell by osmotic shrinkage. When the cooling rate is sufficiently low and the water permeability of the cell is high enough, intracellular ice crystals will not form. When the concentration of dissolved solutes is sufficiently high, the viscosity of the remaining unfrozen water fraction increases and the rate at which water can diffuse into the ice crystal reduces, thereby stopping ice crystal growth. Combined with lowering temperatures the solution will vitrify forming a glassy state. Intracellular injury to the cell can occur, when the cytoplasm approaches homogenous nucleation temperature and freezes and also when the extracellular ice achieves a radius small enough to penetrate the cell membrane pores [20].

Thus, the objective of any good cryopreservation technique is to achieve intracellular vitrification without ice crystal formation. Mazur and Leibo's proposed the "two-factor hypothesis of freezing injury" basis their elegant experiments where they established a quantitative relationship between extracellular and intracellular freezing with respect to cooling rates [21]. Intracellular ice formed at high cooling rates are damaging to the cell, whereas solute concentration at low rates of cooling are lethal to the cell as compared to the innocuous extracellular ice.

3. Cryoprotectants

In 1949, the seminal work of Polge, Smith and Parkes in discovering the cryoprotective effect of glycerol is probably the single most important discovery in the field of cryopreservation [22]. These preliminary experiments were first done with fowl spermatozoa followed successfully by the preservation of bull sperm and later on the successful report of a live birth of calf using Artificial insemination in 1951 [23]. Glycerol still remains the cryoprotectant of choice for most mammalian species. This is despite the fact that glycerol exhibits a higher level of toxicity for sperm when compared to other cell types [24].

Both permeable and non permeable cryoprotectants increase the solute concentration in a given solution, thereby reducing the amount of freezable water. But, as discussed earlier, the rise in solute concentration during ice formation can itself result in cellular injury. Cryoprotectants are not fool proof and the ex-

tent of cell survival depends on 1) the cell line being frozen 2) the cryoprotectant concentration used 3) the cooling rate and warming rate. Different cryoprotectants influence the cooling and warming rates for different cells in many different ways [25].

Thus, a good cryoprotectant can be defined by certain properties as follows

1) The cryoprotectant should be soluble in water and should lower the temperature of freezing. The cryoprotectant should also remain dissolved in water at these low temperatures

2) It should also have a low toxicity to allow the use of a sufficiently high concentration to achieve adequate cryoprotection from intracellular and extracellular ice formation

Conventional techniques of human sperm freezing were developed over a period of 50 years by experimenting with different combination of cryoprotectants and cooling/warming rates. Most current protocols of conventional sperm freezing allows a 50% sperm survival on thaw and the clinical use of frozen thawed sperm is well established in different fertility procedures [26]. However, conventional sperm freezing, even after these 50 years remains unoptimized. Sperm parameters in terms of motility, vitality and DNA integrity is still significantly compromised after conventional slow freezing of human spermatozoa.

The human spermatozoa is particularly sensitive to osmotic shock and low temperatures in the range of 0 degree to 20 degree Celsius. This is termed as "cold shock". This susceptibility varies both between individuals and within individuals in a species [27]. Commercially available sperm freezing solution uses egg yolk to for added protection from cold shock. The exact mechanism of protection is unknown [28].

Another interesting observation in the human sperm is that, these cells do not show the typical "u"-shape of cryosurvival as predicted by Mazur's hypothesis. Sperm also seem to be relatively insensitive when cooled below the freezing point. As compared to other cells, identifying an optimal cooling rate for sperm has been historically difficult [29]. Most conventional semen cryopreservation protocols that involve vapour phase cooling and a combination of different cryoprotectants, diluents and/or different combination of the former have at large only empirically refined the freezing protocol [30]. Sperm survival varies anywhere between 50% to 60% and surviving sperm have been used for both IUI (intra-uterine insemination) and IVF (in-vitro fertilization) worldwide.

Current practice of sperm cryopreservation is of two types used in most ART clinics around the globe. They are static freezing in the vapour phase or the use of multi-step controlled rate non linear freezers. In the static vapour freezing method, semen mixed with an equal volume of cryoprotectant solution is placed in the vapour phase just above the LN2 level. After about 20 minutes, the sample is then plunged into liquid nitrogen.

The use of controlled rate programmable freezers involves the "no seeding" technique to avoid ice nucleation. Samples are first cooled to −4 degree Celsius

at 2 degree Celsius/minute followed by a cooling rate increase to 10 degree Celsius/minute till −100 degrees is reached. This is then followed by plunging into liquid nitrogen [30].

Both freezing and thawing of human sperm have been found to significantly increase the apoptosis markers on the cells. A significant decrease in sperm motility, vitality and mitochondrial activity occurs to membrane lipid peroxidation. Sperm freezing also significantly causes DNA fragmentation, and DNA fragmentation is seen to significantly increase in the first 4 hours after thawing [5].

4. Sperm Vitrification

Vitrification as a cryopreservation technique involves rapid freezing at ultra high cooling rates so that a *glassy state* is achieved without ice formation. Early experiments on vitrification were not successful due to in achievable high cooling and/or warming rates [7]. With the discovery and usage of cryoptotectants along with liquid nitrogen and different cell carrier devices, successful vitrification techniques have been applied to different species of embryo's and a few other cell lines [31]. Vitrification of human embryos is now done routinely in all ART clinics.

Vitrification has 2 major advantages over slow freezing. They are

1) The techniques and methods are quick and easy to learn do not require the use of programmed freezers. This also makes vitrification cost effective.

2) The use of nonpermeating cryoprotectants in vitrification protects the cell from the toxicity of permeating cryotprotectants such as osmotic shock, lipid membrane damage and DNA damage.

Human sperm vitrification was first reported by Nawroth and Isachenko [32]. Different non permeating sugars have been successfully used in the vitrification protocol. This is based on the principle that human sperm cells contain an intracellular milieu that is high in proteins/sugars and low water, thus allowing vitrification to occur as lower temperatures in the absence of permeating cryoprotectants [33].

The use of sucrose as a non permeating cryoprotectant in sperm vitrification was associated with good rates of sperm motility, vitality and mitochondrial membrane potential. The combination of sucrose and albumin in the cryoprotectant solution gave equivalent results in terms of sperm capacitation, acrosome reaction and mitochondrial membrane potential when compared with conventional slow freezing of sperm [34].

In terms of methodology of vitrification, a simple capillary technique of sperm vitrification has been reported. Different techniques that involve use of carrier devices like cryoloop and cell sleeper have also been recently reported [35]. Ischachenko's group suggested directly placing 30 micro litre droplets of semen mixed cryoprotectant into liquid nitrogen. Although, the technique is feasible, and no reported case of viral cross contamination has been reported, the development and use of open pulled straws (OPS) and cut standard straws (CSS) have

further refined the technique of aseptic vitrification [36].

Moskovtsev group also reported excellent recovery in terms of sperm kinematic parameters and DNA fragmentation in a study on 11 infertile men, where they compared slow freezing vs. vitrification of sperm [37]. An interesting finding in this study though, was that although sperm kinematic parameters were found to be better, sperm DNA fragmentation index did not differ between the two groups. Of clinical importance, is the report of live birth of healthy twins after ICSI with vitrified sperm [38]. Sanchez *et al.*, also reported the live birth of after IUI with sperm vitrified without cryoprotectant in a patient with oligoasthenozoospermia, thereby making vitrification feasible for day to day clinical practice [39].

5. Alternate Methods of Sperm Freezing

Different methods of sperm freezing using hamster or human zona have been reported in the literature. While live births have been reported after sperm freezing in human zona or hamster zona, the technical skill required and the cost involved make it unfeasible for day to day clinical practice. Other issues involves the ethical hurdles in using donated human zona or animal genetic material in ART clinics [40]. An interesting alternative method is the mixing of the human sperm with cryoprotectant followed by alginic acid to form microspheres. These agarose microspheres are then vitrified for later use [41]. All the aforementioned techniques give wide variability in terms of sperm survival and motility and this partly depends on the techniques, protocol and quality of sample used.

Freeze dried sperm: Freeze dried sperm (lyophilised sperm) opens up new possibilities in sperm preservation. Freeze drying sperm is based on the principle of *ice sublimation* [42]. Here the prepared sample is first frozen to a solid ice state with the addition of suitable lyoprotectants. The frozen sample is then heated in a lyophiliser under low temperature and low pressure below the critical temperature of water to allow sublimation (direct transition of water from solid state to vapour). The advantage of freeze dried sperm is that the sample can be easily transported over long distances, the freeze drying procedure does not involve the use of cryoprotectant and most importantly all liquid nitrogen based storage hazards are nullified. Freeze dried samples have the advantage of being stored at room temperature.

Successful freeze drying of sperm has been reported in different species of rat, mouse, boar, primate and also recently in human [43]. While freeze dried sperm lose motility uniformly across species, the DNA integrity of the sperm cell is well maintained. The use of chelating agents like EDTA/EGTA and trehalose in the lyoprotective solution is reported to confer protection to the DNA [44]. A few studies have reported live births with the use freeze dried sperm from mouse, rat, rabbit, hamster and horse species [43]. Nevertheless, much more basic research and/or clinical research is required to extend the application of freeze

drying to human ART clinics.

6. Conclusion

While significant progress has been made with different methods of sperm freezing, the single fundamental problem with both slow freezing and/or vitrification is the loss of sperm motility to the tune of 30% - 40% on average. The further development of novel cryopreservation protocols should be based on aggressively researching ice formation kinetics at different cooling rates/warming for the "human sperm". Avoiding the use of permeating cryoprotectant and consequently avoiding the use of liquid nitrogen will be the future of human sperm cryopreservation.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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