The Near Future of Vitrification of Oocytes and Embryos: Looking into Past Experience and Planning into the Future

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Keywords

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Abstract

Currently vitrification is the method of choice for low-temperature preservation of oocytes and embryos. However, that was not the case until about 10 years ago when freezing methods were used relatively successfully for embryos and investigated (unsuccessfully) for oocyte preservation. In this paper we will review the history of oocyte and embryo cryopreservation and look into ways and methods for overcoming and improving the vitrification method since it suffers from inherent disadvantages since it is a cumbersome, time-consuming and costly procedure.

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Introduction

History of Cryopreservation

Cryopreservation, that is the preservation of biological material at very low temperatures, encompasses two main methods: freezing and vitrification. Although freeze drying has been suggested as well, it is still investigational. Freezing is the phase transition of liquid becoming a solid by lowering the temperature to below its freezing point, and vitrification is a phenomenon at which a liquid solidifies without the formation of crystals, thus the process is referred to as a glass transition and the result is an amorphous solid.

Freezing is a natural phenomenon, and anyone who lives in cold areas where it snows in winter has seen freezing occurring in nature all the time. However, vitrification is relatively hidden, and first observations were made only about 200 years ago by the French scientist Joseph Louis Gay-Lussac who discovered the phenomenon of supercooling, that is the maintaining of water at the liquid state although the temperature is below its freezing point. In 1836, Gay-Lussac saw that water that is enclosed in small tubes can be supercooled to -12°C [1]. Several years later in 1858, Johann Rudolf Albert Mousson saw that when spraying water droplets on a cold dry surface, the smaller the droplets the longer they stay supercooled [2]. In 1938, almost a century later, Luyet and Hodapp published the first report on successful cryopreservation of spermatozoa, done by vitrification [3]. In 1940, Luyet and Gehenio [1] wrote that “to avoid freezing, the temperature should drop at a rate of some hundred degrees per second, within the objects themselves” and also “the only method of vitrifying a substance is to take it in the liquid or gas state and cool it rapidly so as to skip over the zone of crystallization temperatures in less time than is necessary for the material to freeze. ... It is evident that when the crystals grow faster one must traverse the crystallization zone more rapidly if one wants to avoid crystallization.” In 1949, Christopher Polge, Audrey Smith, and Alan Parkes [4], when trying to duplicate Luyet’s and Hodapp’s results with fowl spermatozoa, accidentally discovered the cryoprotective property of glycerol and so opened the field of slow freezing while pushing aside vitrification.

And indeed, slow freezing was very successful at freezing sperm leading to the first human baby born using frozen thawed sperm in 1953 [5]. However, embryos and oocytes were more difficult. In 1958, Sherman and Lin [6] showed that mouse oocytes need 8–10 min for equilibration in a freezing solution containing 5% glycerol at 37°C. Such oocytes survived freezing to –10°C and maintenance at that temperature for 3.5 h. In addition, they demonstrated that mouse oocytes survive supercooling to –20°C after slow cooling at 0.6°C/min; however, oocytes that were cooled faster or to lower temperatures were damaged due to crystallization [6].

In the early 1970s, Whittingham [7] had partially succeeded in freezing mouse embryos to –79°C for 30 min using polyvinylpyrrolidone. However, these experiments were not repeated. In 1972, Whittingham, Leibo, and Mazur [8] published the first survival of mouse embryos producing live offspring followed by Wilmut's publication [9] on freezing mouse embryos. The technique included cooling at a slow rate in the presence of 1 mol/L dimethyl sulfoxide (DMSO), which most likely was the ingredient that enabled this. Shortly after, in 1973, Wilmut and Rowson [10] published the first farm animal (a calf) to be born after transplantation of frozen thawed embryos. In 1976, Willadsen et al. [11] froze sheep embryos using 1.5 mol/L DMSO and a cooling rate of 0.3°C/min. Since then, dozens of species have been successfully cryopreserved by slow freezing [for a review, see 12].

Vitrification overshadowed by the success of slow freezing waited until 1985, when the first successful vitrification of mouse embryos using a relatively large volume sample was achieved by Rall and Fahy [13] applying a mixture of DMSO, acetamide, and polyethylene glycol and in a relatively large volume inside a 0.25-mL straw that was plunged into liquid nitrogen (LN). Oocytes were discovered as more difficult to cryopreserve than embryos. This difference in their preservation ability was mainly attributed to difference in lipid compositions between the two, with less polyunsaturated fatty acids in the oocytes compared to embryos [14]. Changes in oocyte membrane fatty acid composition by either nutrition or fusion with liposomes affected biophysical parameters and chilling sensitivity causing increased chilling sensitivity to oocytes [15]. In the late 1980s and early 1990s the “minimal drop size” method was developed by Arav et al. [16, 17]. This method applied the minimal size that maintained oocytes or embryos without damage owing to desiccation. The volume used for vitrification was in the range of 0.07 µL (70 nL), and the concentration of the vitrification solution was about 50% lower than of the vitrification solution used for large-volume vitrification [17]. This method has directly led to the success of oocyte vitrification [18]. It allowed for faster cooling rates and using lower concen-

trations of cryoprotectants (CPs) due to volume reduction [19, 20]. Vitrification is currently producing very satisfactory outcomes by applying the basis of the minimal volume [18, 21].

The Fundamentals of Freezing and Vitrification

As stated above, vitrification is the process in which a sample solidifies without the formation of ice crystals, thus resulting in a glassy amorphous state. It requires high viscosity, high cooling rates, and a small sample volume [20]. The relation between these three factors is explained by Arav's equation:

$$P = CR \times \eta/V,$$

where P = probability of vitrification, CR is cooling or warming rate, η is the viscosity and V is the volume [12]. Freezing, on the other hand, requires nucleating factors that will induce ice crystal growth, either spontaneously as temperature reduces or deliberately by doing what is referred to as seeding [20]. The nucleating agents occur spontaneously in a solution [1], and the lower the volume of the sample, the lower is the chance of such a nucleating agent to occur [1]. When temperature declines and the viscosity increases, the freezing point of a solution decreases; however, at the same time the glass transition temperature of a solution increases. On the basis of this difference, we apply different means to outrun and avoid freezing such as increasing the viscosity (i.e., concentration) and cooling rates while decreasing the sample's volume [16, 17, 19, 20]. The first approach to vitrification focused on increasing viscosity by using high CP concentrations [13]. However, these concentrations are damaging to oocytes [22]. So, the next approach was to increase the cooling rates in order to avoid chilling injury [23] and facilitate a higher cooling rate in order to successfully vitrify oocytes [18, 19, 23]. Many of the successful methods today apply plunging oocytes and embryos into LN in what is referred to as an open system [12, 18], although it poses a potential risk of contamination and cross-contamination [24, 25] due to direct contact with the LN or LN slush [26]. The last approach was to reduce the sample's volume so as to allow reducing the CP's concentration and increase the cooling rate [18–21]. This approach encompasses the advantages of the three requirements for successful vitrification.

Vitrification can be looked on as “the extreme case of supercooled water” [27]. The nucleation rate of intracellular ice crystals is a function of temperature and cytoplasm composition [28]. The classic theory says that a stable ice nucleation forms by random clustering of water molecules [28]. Therefore, nucleation is a statistical occurrence by its nature; thus, the more water molecules presented, the higher the chances for nucleations to occur. Therefore, a key factor in trying to prevent these nucle-

Fig. 1. Illustration of the influence of sample volume, viscosity, and cooling rates on the vitrification of an oocyte. The sample's volume reduction reduces chances for ice nucleations to form and increases cooling rates, high viscosity prevents both the formation of ice nucleations and the advancement of ice crystals once formed thus preventing flushing, and the high cooling rates also decrease ice crystal growth. If these three parameters are not fulfilled, nucleation, crystallization, and flushing occur which results in cell death.

ations will be the sample's volume. Adrian et al. [27] showed that a thin layer of less than 1 μm of pure water or dilute aqueous solutions results in vitrification when it is immersed in liquid ethane or propane. The success of vitrification was attributed mainly to the high cooling rate. However, this is an incomplete explanation, since once an ice nucleation is formed, the ice crystal propagates at a very high velocity. As mentioned above the chances for ice nucleations to occur is correlated with the sample's volume [28] and according to Turnbull's equation [29, 30] the velocity at which the ice crystals advance (u) in a viscous solution is inversely correlated with the viscosity (η) and directly proportional to the function of the system's supercooling ($f(\Delta T_r)$). It can be plotted as follows:

$$u \cong \frac{Ku}{\eta} f(\Delta T_r),$$

and Ku is a constant determined by the designed model [31].

Figure 1 shows an illustration of how the three factors of viscosity, volume, and cooling rate affect vitrification prospects in an oocyte. From this illustration we can see that the viscosity is the most important factor since it affects both the chances for ice nucleation to occur as well as the ability to progress afterwards. The sample's volume influences the chances of ice nucleations to form as well as the cooling rate, and the cooling rate affects the ability of the crystals to progress (Fig. 1).

Mazur [32] found that if ice crystals occupy 16% of the oocyte they will cause cell death. That means that if we want to prevent a cell's death it is best to avoid intracellular crystallization at all. Therefore, we have calculated that the cooling rate should be more than $450,000^\circ\text{C}/\text{min}$ to avoid intracellular crystallization in a human oocyte loaded with 10% DMSO and 10% fetal calf serum if ice nuclei exist [Arav, unpubl. data]. Therefore, when trying to evaluate the importance of each factor on the chances for vitrification, the cooling rate is probably not the main factor in avoiding crystallization since reaching such a high cooling rate is almost impossible. However, increasing the viscosity and decreasing the volume, which are easier to do, are more important for achieving vitrification.

How Understanding Fundamentals Advances the Field

Understanding how vitrification works, what influences it, and what kind of damage may occur during the processes of vitrification and warming have led to several developments in the field.

Among the first developments were evaluating different CPs after the first to be discovered, i.e. glycerol [12]; the next were: DMSO [22], propylene glycol [33], ethylene glycol [34], antifreeze proteins [35, 36], polyvinylpyrrolidone, sugars (sucrose, glucose, etc.) [22], proteins (albumin, starch, etc.). Then, after the minimal drop size

had been published, many developments based on this principle were developed for vitrification of oocytes such as the Cryotop, CryLeaf, etc.; for a review, see Willadsen et al. [11].

The sample's volume reduction also led to reducing concentrations of CPs, which are known to cause osmotic stress and are toxic at high concentrations to many cells [22].

Trying to increase the cooling rates was one of the most difficult and still is a challenging factor to overcome. The use of LN leads, of course, to high cooling rates; however, when a sample is inserted, it causes the LN to boil and there is an insulating vapor phase surrounding the sample until it reaches the LN temperature [20]. A device named Vitmaster (IMT, Nes-Ziona, Israel) was developed to overcome this hurdle by applying negative pressure on the LN reducing its temperature almost to -210°C , thus receiving LN slush and avoiding the boiling of the LN when the sample is inserted [20].

As well as trying to solve problems that are associated with the vitrification/warming processes directly, other problems have risen due to the practicality of the procedures. One of the problems is the risk of contamination and cross-contamination [24, 25] through the LN, especially when it is done in an open system which was proven to have superior results compared to a closed system with regard to oocyte vitrification [37, 38]. There are currently several devices that offer clean LN such as the Elan 2 (MMR Technologies, San Jose, CA, USA) which produces clean LN, the Nterilizer[®] (Nterilizer, Bologna, Italy) which sterilizes the LN using ultraviolet light [39], and the Veriseq[®] (Linde Gas, Schiedam, Holland) which filters gaseous nitrogen, liquifying it to produce sterile LN. We have developed our own system which simply creates sterile liquid air having the sample temperature as LN (-196°C) by liquifying following air filtration [26].

Furthermore, one of the problems that evolved due to the toxicity of the CPs is the necessity to transfer the oocytes or embryos between several solutions with increasing concentrations until reaching the final vitrification

solution prior to plunging into LN, and upon warming there is the same need but with declining concentrations of sugars. This has made the process cumbersome, much more costly, highly skilled, and causes differences between centers [40–42]. The need to standardize the process is crucial. There are relatively new talks about automatizing the process; there is the Gavi (Genea Biomedx, Sydney, Australia) system which is mainly for embryos since the cooling rates that it produces are rather low for oocytes [41]. We have also developed a system that automatizes the process including the insertion into LN and the warming process named Sarah (Fertilesafe, Nes-Ziona, Israel) [41]. Using this device we vitrified oocytes and embryos successfully [41], as well as reproductive and nerve tissue [43]. There are also new publications regarding robotics systems for embryo vitrification [44]. Most likely future developments will proceed towards the automatization of the process although now these processes seem mainly to mimic the work that is currently done by the embryologists and not changing the paradigm.

Statement of Ethics

The authors have no ethical conflicts to disclose.

Disclosure Statement

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Author Contributions

Arav Amir has done the experiment and together with Natan Yehudit wrote and edited the paper.

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