New trends in gamete’s cryopreservation

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Abstract

We developed new techniques to improve freezing and vitrification of sperm, oocytes and embryos. Our novel freezing technology is based on ‘Multi-Thermal-Gradient’ (MTG) freezing that is used for sperm. The freezing apparatus has the ability to control ice crystals propagation by changing thermal gradient or the liquid–ice interface velocity which optimizes ice crystals morphology during freezing of cells and tissue. Using this apparatus we were able to freeze bull, stallion, boar, ram, fowl and human sperm with normal post-thaw motility/pre-freezing motility of 70–100%. The vitrification method includes the cooling of nanoliter sample (the ‘Minimum Drop Size’ technique) in ‘super-cooled’ liquid nitrogen (−210 °C), which maximized cooling rate to the highest physically possible (24–130 000 °C/min). Using this method we achieved very high survival of bovine oocytes and embryos. Vitrification of oocytes at the MII stage resulted with cleavage and blastocyst rate of 50 and 20%, respectively. The vitrification of in-vitro production (IVP) of bovine embryos allowed the production of a healthy calf after embryo-transfer carrying the name ‘Zegugit’ (in Hebrew: made from glass). © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Developments in applied reproduction technologies in the 20th century stimulated many scientific breakthroughs in cryobiology. This was because of the need for preservation of the gametes so that they would be available for the required technology. The first technology to be developed, at the beginning of the last century was artificial insemination (AI). Twenty years later Polge discovered the first cryoprotectant (glycerol) and achieved the first successful cryopreservation of sperm (Polge et al., 1949). Following AI the first cattle born after surgical embryo transfer (ET) was reported by Willett in the 1950s (Willett et al., 1951). This, too, was followed by a 20-year interval before the first freezing of mouse embryos, achieved by Wittingham and colleagues (Whittingham et al., 1972). The first cattle in vitro fertilization (IVF) was performed at the beginning of the 1980s (Brackett et al., 1980) and, following another 20-year interval, soon after the first cloning (Wilmut et al., 1997) and successful cryopreservations of cattle oocytes have been reported in several publications (Arav et al., 1993a,b Arav and Zeron, 1997; Fig. 1; Vajta, 2000; Kuleshova et al., 2000).

However, independently of the reproduction technologies, cryobiology was developed from the beginning of the 20th century, following the finding that intracellular crystallization and chilling injury were major causes of cell death at low temperatures (Molisch, 1897). Luyet and Hodapp (1938) were the first to demonstrate successful cryopreservation of frog sperm by vitrification (ice-free rapid solidification) Polge et al. (1949) aiming to repeat Luyet results, discovered the cryoprotective property of glycerol and so opened the field of slow freezing.

Currently, there are two methods for gamete cryopreservation: slow freezing and vitrification. Slow freezing has the advantage of using low concentrations of cryoprotectants (CPs), which are associated with chemical toxicity and osmotic shock. Vitrification is a rapid method, which decreases chilling injury (cold shock) and requires only a brief procedure.

2. Vitrification

Vitrification is a process by which liquid turns into solid without the formation of ice crystals (Luyet and
Hodapp, 1938). Three factors affect the probability of vitrification: cooling rate, viscosity and volume. Increasing the viscosity or cooling rate, or decreasing the volume will each increase the probability of vitrification. In the past, vitrification was achieved simply by plunging the sample into liquid nitrogen (LN) at −196 °C (Rall and Fahy, 1985). In this method, heat transfer from the sample into the LN leads to the evaporation of LN around the sample, resulting in the formation of a nitrogen gas layer, which acts as an insulator. This insulation reduces the heat transfer and makes it impossible to achieve uniform and rapid cooling rates.

We have developed a new device (Vit-Master®, IMT, Israel) whereby the temperature of the LN is reduced to as low as −210 °C by applying negative pressure to it (Arav, 1998). Liquid nitrogen slush is then formed and the cooling rate is dramatically increased. The cooling rate is especially enhanced in the first stage of cooling (from 20 to −10 °C), when it is six, four or two times higher with 0.25-ml straws, open pulled straws (OPS) (Vajta, 2000) or electron-microscope (EM) grids (Steponkus et al., 1990), respectively. Between −10 and −150 °C, the cooling rate is only about doubled by use of the Vit-Master, but that was found to be enough to reduce the chances of devitrification and recrystallization during warming. Recent publications show that the use of new containers such as EM grids (Steponkus et al., 1990; Martino et al., 1996; Arav and Zeron, 1997), OPS (Vajta, 2000) decreases the volume of the solution, increases the cooling rate and results in successful vitrification of oocytes and embryos. In the past, researchers designing optimal protocols for vitrification of oocytes and embryos, have focused on developing solutions with higher viscosity, by using high concentrations of permeating (glycerol, DMSO, propylene glycol, ethylene glycol) or non-permeating (Ficoll, sucrose, trehalose, PVP) CPs. However, the CPs used to increase viscosity are often damaging because of their toxicity or osmotic effects. Furthermore, decreasing the volume of the solution, reduces the probability of ice crystal formation and allows successful vitrification in the presence of low (and non-toxic) concentrations of vitrification solutions.

Using this principle, we (Arav, 1992; Arav et al., 1993a,b; Arav and Zeron, 1997) have developed a new technique known as the 'minimum drop size (MDS)' technique, in which nanoliter (0.1–0.5 µl) volumes of vitrification solution are cooled and warmed. The MDS technique has been successfully used to vitrify pig (Arav et al., 1993a,b) and bovine oocytes (Arav and Zeron, 1997), and cow and sheep embryos (Arav et al., 2001).

A modification of the MDS technique was developed by us very recently and will be describe here: we placed 0.5-µl drops on cover slip glass slides measuring 1.5 mm wide, 3 cm long and 0.1 mm thick (Fig. 2a). In Fig. 2b, we see all the possible outcomes when these drops are cooled in liquid nitrogen: the left-hand photograph shows darkness of the drop, indicating crystallization of ice during cooling, while the right-hand one shows glass fractures. In the bottom photograph, however, the drops remained transparent, with no crystallization or fractures, despite the reduction of CP concentration by 50%. We conclude that the combination of increased cooling rate with reduced volume is what is needed to ensure a high probability of vitrification in drops containing low concentrations of vitrification solution (50–75% of the original concentration).

Other problem associated with vitrification are the fractures of the glassy solution. When we plot the probability of fractionation against the concentration of the CPs, the volume of the drop and the cooling rate, we see that the fractures appear only when the volume is above 1 µl (Fig. 3). Surprisingly, at 1 µl, fractures appeared when the concentration of the vitrification solution (VS) was high (100% VS = 38% ethylene glycol, 0.5 M Trehalose and 4% BSA in TCM medium). At the half-concentration of VS (50% VS), fractionations were observed only at a high cooling rate. We suggest here a simple explanation of this phenomenon, based on the following equation:

\[
\text{Probability of fractionation} = CRX\frac{\Delta T}{V}
\]

where CR is the cooling rate, \(\Delta T\) is temperature difference (between glass transition and LN temperature) and \(V\) is the volume.

The reason for the increasing probability of fractionation in high concentrations of VS is thought to be related to the glass transition temperature (Tg). We know that fractions can form only at temperatures below that at which the liquid turns into glass (Tg) and above the LN temperature (−196 °C). We also know that a solution with a higher concentration will have a higher Tg. Therefore, if the temperature gradient increases, as in the case of higher Tg, then the probability of fractionation will also increase.

![Fig. 1. Developments in applied reproduction technologies and its effect on cryopreservation.](image-url)
Finally, the results of vitrification of bovine oocytes at the MII or GV stage, with a concentration of 75% VS, have been reported elsewhere (Arav et al., 2000). We achieved 72 and 38% cleavage and blastocyst rates formation, respectively, for the vitrified MII oocytes and 27 and 14% cleavage and blastocyst rates formation, respectively, for oocytes vitrified at the GV stage.

We conclude that the new vitrification procedure, which features small volumes, direct contact with supercooled LN and low concentrations of VS, reduces chilling injury and provides a high probability of vitrification in the absence of glass fractures.

2.1. Freezing of sperm

Heat transfer in cells, tissues or organs, which have larger volumes (i.e. more than 0.1 ml), is too slow to permit vitrification without risk of ‘solution effect’ or crystallization. It is therefore preferable to freeze large volumes such as semen and tissue by means of slow cooling rate procedures (0.5–100 °C/min). This method requires only low concentrations of CPs and is characterized by a more stable thermodynamic equilibrium (the presence of ice crystals vs. glass).

When a biological sample containing living cells in a freezing solution is being frozen, the first portion of the sample to freeze is the intercellular water. During slow freezing the cooling rate is thought to be the major factor that determines the survival of cells and tissue. The rate of cooling also affects the morphology of the intercellular ice crystals (Mazur, 1966): morphologies such as closely packed needles kill cells by external mechanical damage (Arav et al., 2000, unpublished results). Thus, maximizing the survival rate of cells subjected to freezing and thawing requires careful control of the freezing process.
Fig. 3. The effect of volume, cooling rate and VS concentration on the probability of 'glass' fractures.

Table 1
Survival of sperm after freezing with MTG system

<table>
<thead>
<tr>
<th>Species</th>
<th>Fowl</th>
<th>Ram</th>
<th>Bull</th>
<th>Stallion</th>
<th>Boar</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>30</td>
<td>6</td>
<td>45</td>
<td>15</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Post-thaw motility (%)</td>
<td>45</td>
<td>57</td>
<td>65</td>
<td>50</td>
<td>45</td>
<td>65</td>
</tr>
</tbody>
</table>

The conventional slow-freezing method involves lowering the temperature of the chamber in a controlled stepwise manner. This method is based on using multidirectional (equiaxial) heat transfer to achieve a rate of temperature change in the sample that depends on the thermal conductivity and geometrical shape of the container and of the biological material within it (Diller, 1992). The thermal gradient within the sample is determined implicitly by the temperature of the chamber and the thermal conductivity of the materials of the sample, and is not directly controllable.

Furthermore, the ambient temperature gradients within the freezing chamber and the unreliability of temperature recording measurements (Koebe et al., 1993) add to the difficulty of achieving the optimal cooling rate.

A new freezing method, ‘Multi-Thermal-Gradient’ (MTG®) (Arav, 1999) aims to overcome the above-mentioned problems and to enable the cryopreservation of large-volume samples.

This freezing technology is based on directional freezing; the biological material is moved through a linear temperature gradient so that the cooling rate and ice front propagation are precisely controlled. This method also enables the incorporation of controlled seeding into the freezing process. When any liquid is cooled below its freezing point, it remains a liquid, in an unstable super-cooled state, until freezing starts at randomly distributed nucleation sites and spreads throughout the entire volume of the liquid. As discussed above, in the conventional equiaxial method of freezing, ice grows with uncontrolled velocity and morphology, and may disrupt and kill the cells of the samples.

Ideally, the velocity of the freezing front should be such that the ice morphology does not disrupt the cells or tissue. However, the rate of cooling appropriate for favorable ice morphology may not be appropriate for other desired outcomes of a sample’s freezing protocol. The laterally varying gradient used in our technology allows cooling to proceed at differing rates under varied temperature regimes, thereby facilitating full control over nucleation and ice crystal morphology.

We applied this method to sperm of various species—fowl, ram, bull, stallion, boar and human—and the results are summarized in Table 1.

It is concluded that this technique could be very effective for sperm freezing of different species including human.
References


Arav, A., Zeron, Y., 1997. Vitrification of bovine oocytes using modiﬁed minimum drop size technique (MDS) is effected by the composition and concentration of the vitriﬁcation solution and by the cooling conditions. Theriogenology 47, 341–342.


