

*KrioBlast*TM as a New Technology of Ultrafast Cryopreservation of Cells and Tissues. 2. Kinetic Vitrification of Human Pluripotent Stem Cells and Spermatozoa

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Pilot experiments on kinetic vitrification of human pluripotent stem cells and spermatozoa using a *KrioBlast*TM-2 without penetrating cryoprotectants have shown high survival of cells (75-85% in both cases).

Key Words: *cryopreservation; kinetic vitrification; Leidenfrost effect; reproductive medicine; regenerative medicine*

Vitrification is essential for long-term biopreservation (stabilization) of biological structures [1]. Vitrification agents (VFA) are the substances penetrating and not penetrating into the cell and playing an important role in vitrification processes. In most of the existing methods of the so-called equilibration vitrification, very high concentrations (penetrating VFA) are required. These VFA can lead to serious mechanical damage to the membrane and cellular structures due to osmotic imbalance and to latent chemical disorders of the epigenetic systems responsible for cell development and differentiation. Kinetic vitrification (KVF) with the use of cryogenic agents is one of the most promising methods of biopreservation, because it does not require potentially toxic and osmotically active penetrating VFA [1].

KVF of cells in most cases requires extremely high rates of cooling to cryogenic temperatures (hundred of thousand °C/min). The exception is the sperm of humans and some animal species, which was described in early studies and in modern reports [3-5].

Review of published reports and the key aspects of thermodynamic processes pertinent to KVF are pre-

sented in our previous paper [1] that describes a platform for hyperfast cooling *KrioBlast*TM-1 employing liquid nitrogen (LN₂). The Leidenfrost effect is the main obstacle in attaining hyperfast cooling rates, but the use of this platform and first generation containers for KVF *VitriPlate*TM-1 enabled vitrification of a considerable volume of 15% glycerol (4000 μl), which corresponded to cooling rate of 100,000-200,000°C/min [9]. However, this platform requires highly qualified personnel, which limits its use outside the laboratory.

Here we present an optimized semi-automatic platform *KrioBlast*TM-2 and preliminary results of KVF of pluripotent stem cells and human spermatozoa.

MATERIALS AND METHODS

Semi-automatic platform for kinetic vitrification *KrioBlast*TM-2. Using a manually operated pilot device *KrioBlast*TM-1 [1], we achieved vitrification of up to 4000 μl 15-16% glycerol solution, which corresponded to the cooling rate of 100,000-600,000°C/min. The cooling rates and vitrification volumes for more dilute solutions are limited by the following factors: the sample was plunged manually using a plunger [1]; LN₂ stream velocity in the cryogenic “Charcot’s douche” was insufficient for complete elimination of the Leidenfrost effect [1]; the plate holding the sample was covered with plastic and additional time was re-

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quired for sample unwrapping, which led to partial devitrification. All these drawbacks were eliminated in the second semi-automated version *KrioBlast*^{TM-2} designed for hyperfast cooling of samples (Fig. 1).

KrioBlast^{TM-2} consists of a pressurized LN₂ source (1), cooling chamber (2), and Dewar's flask (3). High pressure in tank (1) is created with an adjustable power supply for the electric heater (4). Valve (5) is positioned on the high-pressure tube with LN₂ connecting tank (1) and cooling chamber (2). Valve (6) is positioned on the tube with LN₂ connecting cooling chamber (1) and Dewar's flask (2). Cryogenic container VitriPlates^{TM-2} (cryogenic plate) contains a "biological substance" — marker (7) and LN₂ (8) for its cooling. Plate (7) is cooled under a "shower" — LN₂ sprayer (9). The system contains a normal pressure discharge pipe (10), high pressure discharge pipe with a pressure relief valve (11), a safety valve for gaseous nitrogen (12), and a pressure gauge (13). For rapid introduction of the cryogenic plate (7) into the cooling chamber (2) under LN₂ sprayer (9), a "Speargun" plunger (14) with a pushing spring (15) is used. The cooling chamber (2) contains a barrier to prevent LN₂ splashing (16), a window for cryogenic plate (17), a side observation window (the lower illumination window is not shown) (18) for visual control (and photographing) of cooling of the plate with the biological substance (7) under LN₂ jets.

Cryogenic plates VitriPlates^{TM-2} are designed for evaluation of the critical cooling rate ensuring vitrification. The cryogenic plates were filled with different concentrations of the marker (glycerol in H₂O) and sealed. General view of *KrioBlast*^{TM-2} system, cryogenic containers VitriPlates^{TM-2} with glycerol, and Speargun plunger is presented in Figure 2.

Estimation of the cooling rate of marker solutions in *KrioBlast*^{TM-2}. The cooling rate of the biological sample was determined by monitoring vitrification

vs. ice formation at different glycerol concentrations. Critical cooling rates of aqueous glycerol solutions are well known for a wide range of concentrations [9]. Cooling rate of the biological sample in *KrioBlast*^{TM-2} was determined in the same way as in *KrioBlast*^{TM-1}. Glycerol solutions with concentrations of 6-16% with a step of 1% were prepared. The previous most reproducible result was obtained with 16% glycerol. During cryogenic cooling, either vitrification (transparency) or crystallization (opacity) of the sample was observed. However, some researchers argue that optically clear samples can contain ice crystals, as this deviation corresponds to transition from vitreous to microcrystalline state, and limits any discrepancy between the optical- and X-ray-deduced transitions to less than 2% in glycerol concentration [9]. The maximum cooling rate was estimated from the minimum glycerol concentration that can still be vitrified using a correlation curve [9] (marker solutions did not contain cells, glycerol was not used for KVF of cells).

Kinetic vitrification of pluripotent stem cells and spermatozoa. Preliminary experiments on vitrification of human embryonic stem cells (hESCs, provided by Prof. E. Y. Snyder, Dr. Y. Liu, and Dr. D. Wu) were carried out at the Stem Cell Center at the Sanford Burnham Prebys Medical Discovery Institute (SCC SBPMDI). Human spermatozoa obtained from healthy donors at the Center for Human Reproduction at the University of California San Diego were provided by Prof. S. Agarwal. The biological samples were subjected to KVF and plunged in LN₂ for at least 2 h. Then, the samples (cell suspensions in sealed VitriPlate^{TM-2} cryogenic plates) were warmed in a water bath at 37°C and transported to SCC SBPMDI for analysis. Cell viability was assessed by trypan blue staining on an automatic cell counter (Invitrogen). Trypan blue staining allows accurate assessment of

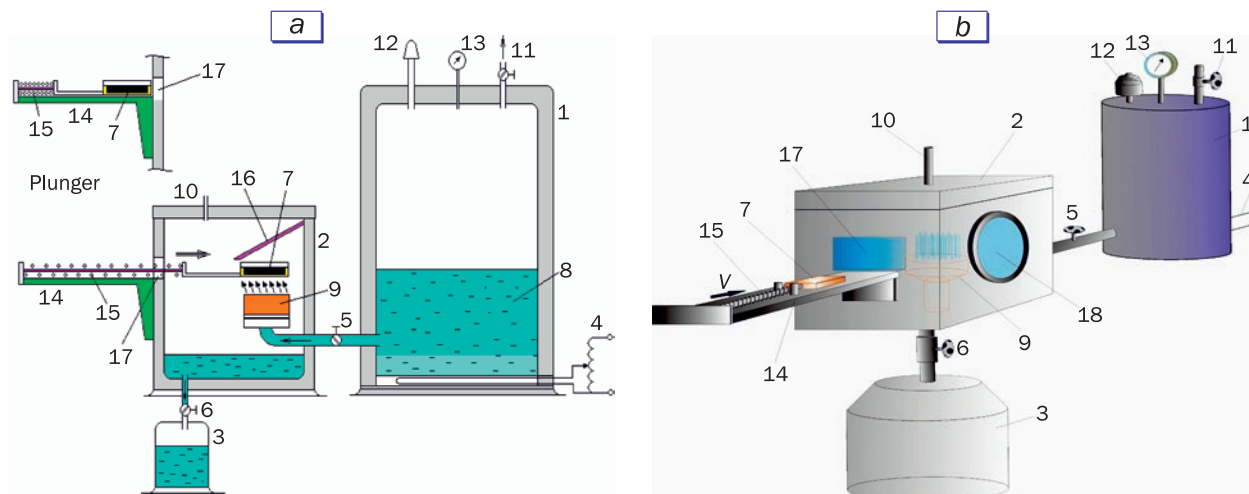


Fig. 1. *KrioBlast*^{TM-2} platform: scheme (a) and 3D configuration (b).

the level of cell death associated with damage to the cell membrane and decomposition and fragmentation of cells. For KVF, the cells were suspended in PBS (spermatozoa) and standard culture medium for stem cells adopted in SCC SBPMDI supplemented with 0.5 M sucrose.

The cells were divided into the following groups: 1) intact control (cell viability was evaluated in medium not supplemented with 0.5 M sucrose); 2) control without freezing, but with the use of Speargun plunger (cells in medium with 0.5 M sucrose were introduced with the plunger into the cooling chamber pre-cooled to cryogenic temperatures); 3) KVF “T→T”: samples were warmed (defrosted) in a water bath and remained transparent during both rapid cooling and heating; 4) KVF and thawing in air “T→O”: samples were transparent in LN₂, but became opaque during slow warming in air due to (re)crystallization; 5) “O”: samples were manually plunged in LN₂ without hyperfast cooling with LN₂ jets and then warmed in a water bath (despite relatively rapid warming, the samples remained opaque due to the presence of ice crystals until complete thawing at temperatures above 0°C).

RESULTS

Estimation of sample cooling rate in *KrioBlast*TM-2. We achieved stable and reproducible vitrification of 12% glycerol, which was observed at 2-fold higher cooling rate, as the rate exponentially depends on concentration of the solution [9]. In some cases, vitrification of 8-10% glycerol solutions was observed, and even vitrification of 7% glycerol solution was once achieved.

However, the absence of technical possibility of precise detection of vitrification, cooling chamber limitations, and most importantly, difficulties in manual transfer of VitriPlateTM-2 containers to water bath reduced reproducibility of results at glycerol concentrations <12%. However, vitrification of 1- and 4-ml sam-

ples was achieved. Hence, semiautomatic high-speed system of sample delivery to the cooling chamber with a Speargun plunger made the cooling process more efficacious and easily controllable.

Kinetic vitrification of cells. The results of pilot experiments on vitrification of human pluripotent stem cells and spermatozoa are presented in Figure 3.

Neither addition of sucrose, nor the use of Speargun for cell delivery induced appreciable damage to the cells in comparison with intact control. Initially lower viability of control spermatozoa (group 2) in comparison with embryonic stem cells (74.0 ± 5.7 and $90.0 \pm 3.6\%$, respectively) can be explained as follows. Stem cells represent a more homogenous population of cultured cells, while spermatozoa of human ejaculate are a very heterogeneous population of cells of different ages. Samples of intact stem cells were examined almost immediately after isolation, while sperm samples were first transported from UCSD to SCC SBPMDI, which increased the time from material collection to analysis. Moreover, thawed cells were exposed to 0.5 M sucrose for 15-20 min. Obviously, spermatozoa could be more sensitive to almost 3-fold hyperosmotic environment. Although the values obtained for group 2 spermatozoa were normalized relative to intact control values, the interval between ejaculation and sample testing could contribute to the lower survival rate of spermatozoa compared to stem cells. Our previous experiments showed that human spermatozoa tolerated this short-time moderate hyperosmotic exposure (0.5 M sucrose for 15-20 min), therefore, it seems unlikely that sucrose affected spermatozoa more deeply than stem cells. It can be also hypothesized that high acceleration and especially abrupt deceleration can create hydraulic shock waves, cavitation, or other physical effects that can lead to cell damage. However, our experiments revealed no statistically significant decrease in the number of cells in the sample after its introduction to the cooling chamber with a Speargun



Fig. 2. General view of *KrioBlast*TM-2 system (a), cryogenic containers VitriPlatesTM-2 filled with different concentrations of glycerol (b), and Speargun plunger in the “released” position (c).

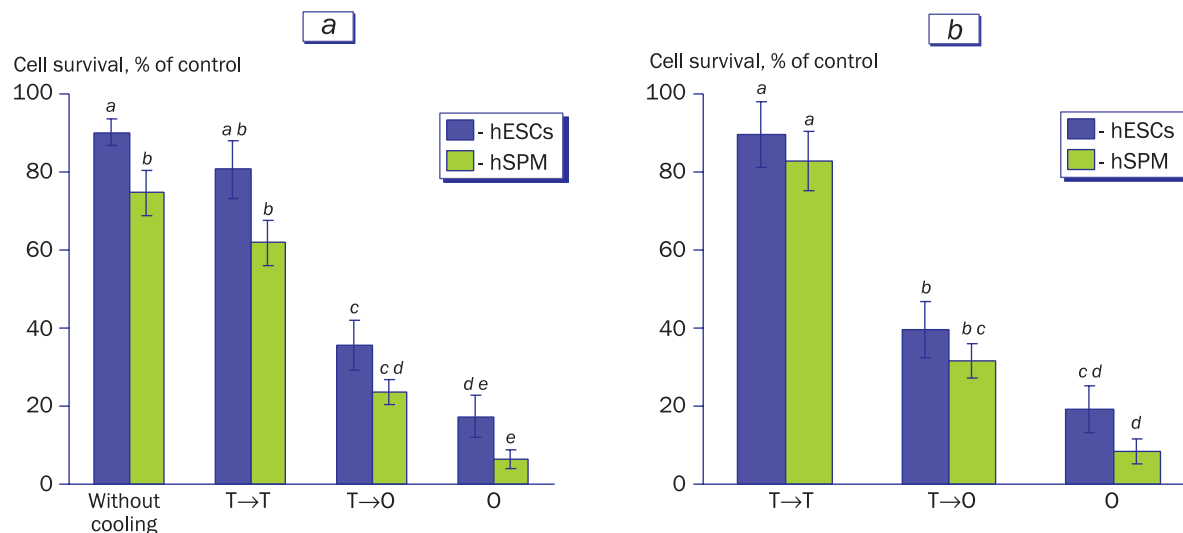


Fig. 3. Viability of human pluripotent (embryonic) stem cells (hESC) and spermatozoa (hSPM) after hyperfast semiautomatic KVF in 0.5 M sucrose solution with the use of Speargun plunger and manual warming on a water bath at 37°C. “T” and “O” are transparent and opaque samples, respectively, during cooling and warming. The values were standardized to intact control (a) and control cells not subjected to cooling (b). The columns marked with the same letters did not differ statistically at $p=0.05$.

or any damage and we did not observe cell debris, detached tails, and other signs of mechanical destruction of spermatozoa. Hence, time delay is probably the main factor affecting the viability of spermatozoa in samples that were not subjected to cooling and subsequent warming.

To evaluate the effect of KVF and warming *per se*, the values obtained in the experimental group were normalized relative to the values in non-frozen samples (Fig. 3, b). When hyperfast cooling resulting in KVF was followed by fast (not necessarily hyperfast) warming not accompanied by devitrification of the suspension, the biological samples remained transparent throughout the cooling and warming cycle (T→T). Cell viability remained high under these conditions, so this cryopreservation cycle did not introduce appreciable damage to either cell type.

The viability index of kinetically vitrified stem cells and spermatozoa (T→T) did not differ statistically. This result was confirmed by normalization of the values to survival rate in the control (group 2), *i.e.* cells not subjected to cooling: T→T 88% for stem cells and 83% for spermatozoa (Fig. 3, b).

At the initial stages of the study, the cell protocols were not optimized. Nevertheless, the examined samples demonstrated high survival rate in comparison with aliquots not subjected to cooling. These results substantiate the need in hyperfast cooling at the cellular level.

If warming of the cold biological sample was performed slowly in air, the sample that was transparent in LN₂ turned opaque (T→O) due to devitrification. In this case, only 25-30% frozen cells were not stained

with trypan blue compared to the control cells not subjected to freezing (group 2).

Slow cooling (not fast enough for KVF) could be achieved by either sample plunging in LN₂ in a foam box or by cooling in LN₂ vapor without douching in LN₂ jets in the cooling chamber. In both cases, crystallization of the sample was observed at the stage of cooling and it remained opaque throughout the thawing process until complete melting of ice crystals (“O”; Fig. 3). In this case, no motile spermatozoa were seen under the microscope, and practically 100% cells were stained with trypan blue irrespective of whether thawing was performed in a water bath or in air.

Thus, the “universal cryopreservation protocol” can be implemented without the use of penetrating, and therefore potentially toxic, exogenous cryoprotectors, because the cells contain endogenous intracellular VFA in sufficient amounts and the culture media usually contain sugars and proteins that are also effective VFA [2,6,8]. In most cases, this universal cryopreservation protocol implemented using kinetic vitrification approach requires hyperfast cooling at cryogenic temperatures. Once such cooling rates are achieved for the most water-rich cells, this protocol will be universal for cells containing less intracellular water. The limiting factor for hyperfast cooling is the Leidenfrost effect (boiling LN₂ on the cooled surface forms a heat-insulating vapor film surrounding the biological sample — film boiling regimen).

For elimination of the Leidenfrost effect, the samples should be cooled with high-pressure LN₂ jets rather than plunged into cryogenic refrigerant. LN₂ impact pressure breaks the vapor film on the cooled surface and

ensures its cooling. This resembles fast cooling of very hot surface with high-pressure water jets. For instance, some industrial devices are cooled by high-speed coolant jets under pressure, or in cryosurgery, skin cells can be frozen and destroyed (although this effect is achieved by a mixture of liquid and gaseous nitrogen).

In our opinion, a crucial role in KVF is played by high-speed delivery of biological sample under high-pressure LN₂ jet, because the sample is not in the zone of gaseous nitrogen, which provides insufficient rate of cooling, and hence crystallization during at the stage of cooling. This is seen from experiments with cells with addition of 0.5 M (18%) sucrose, when the samples became opaque due to crystallization at the stage of cooling. Even in case of fast thawing, the vast majority of cells died.

During hyperfast cooling of samples in *KrioBlast*TM-2, the cell samples became opaque during the subsequent slow thawing. Thus, a significant number of stem cells and sperm cells (70-75% of not cooled cells) died due to devitrification, and probably, recrystallization. In order to distinguish between these two processes, additional studies and special expensive equipment are required.

Thus, equally successful results of KVF for cells of different size, shape, water content, and physiological peculiarities were achieved in this study. Stable and operator-independent KVF results can be achieved with an automatic system excluding contact of the biological sample with air or nitrogen from the moment of cooling to complete rewarming. This three-module *KrioBlast*TM-3 platform with more sophisticated cryogenic container has been patented in the USA and the Russian Federation [2,7], and construction of this system is now in progress.

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