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KrioBlast™ as a New Technology of Hyper-fast Cryopreservation of Cells and Tissues. Part I. Thermodynamic Aspects and Potential Applications in Reproductive and Regenerative Medicine

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Kinetic (dynamic) vitrification is a promising trend in cryopreservation of biological materials because it allows avoiding the formation of lethal intracellular ice and minimizes harmful effects of highly toxic penetrating cryoprotectants. A uniform cooling protocol and the same instruments can be used for practically all types of cells. In modern technologies, the rate of cooling is essentially limited by the Leidenfrost effect. We describe a novel platform for kinetic vitrification of biological materials *KrioBlast*™ that realizes hyper-fast cooling and allows overcoming the Leidenfrost effect. This opens prospects for creation of a novel technology of cell cryopreservation for reproductive and regenerative medicine.

Key Words: *cryobiology; cryobanks; kinetic vitrification; Leidenfrost effect; reproductive and regenerative medicine*

Cryopreservation of gonocytes (germplasm) and especially adult gametes and early zygotes, is the key stage in human reproductive technologies (fertility clinics and cryobanks of the sperm and oocytes), in agriculture (centers of reproduction of elite animals), and in preservation of genetic diversity of wildlife (Frozen zoos) [16]. It is known that cells cannot live long in liquid media, while large ice crystals emerging in the cell during freezing can cause cell death [14]. The only way to store biological materials, in particular cells, for a long (practically infinite) time is to keep them in a glassy (vitreous) state. In this state, viscosity of the cell content and its closest microenvironment in non-frozen channels is very high (comparable with glass viscosity $10^{13.6}$ Pa×sec) and the reactions of degradation are completely inhibited.

Vitrification is the key condition of long-term storage of biological material. At present, cell vitrification can be achieved by five methods [7]. The most common method of vitrification in cryobiology is slow, often programmable freezing when the bulk of extracellular water is transformed into ice, while intracellular water escapes the cell and finally, dehydrated cells are vitrified in a highly concentrated solution (brine) in non-frozen inter-ice channels.

The second widespread method of vitrification implies the use of thickening agents (vitrificants) penetrating into cells. These excipients in high concentrations (by one order of magnitude higher than the isotonic concentration) considerably increase viscosity of the intracellular and intracellular medium enabling vitrification even at relatively low cooling rate [5,19]. This type of ice-free vitrification that after a certain limit did not depend on cooling rate can be called equilibrium vitrification (E-VF), because it is free from both intracellular and extracellular ice. These thickening excipients in E-VF process are erroneously called cryoprotecting agents (CPA). However, this term is

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applicable only to slow cooling where CPA play a role of an osmotic buffer preventing excessive dehydration of cells during crystallization of the intracellular ice.

The method of kinetic vitrification (K-VF) requires much less or no exogenous vitrificants, but manifold higher cooling rates. It also does not require complete vitrification of the extracellular media, because only intracellular vitrification or, at least, the formation of small-crystalline intracellular ice that is non-lethal for cells, is an obligatory condition for cell survival. The principal differences between E-VF and K-VF were previously described by us in detail [7].

These three methods of vitrification form the basis for modern technologies of cryogenic preservation of cells, components, and equipment. Vitrification at high (room) temperature can be realized by two methods: lyophilization (primary drying via ice sublimation under high vacuum and secondary drying at temperatures above 0°C) and xeropreservation (drying at temperatures above 0°C under vacuum, in air, or in an atmosphere of inert gas during the whole cycle). The latter two methods are used for stabilization of viruses and prokaryotic cells, but inapplicable to eukaryotic cells; therefore, cryogenic methods are still the mainstream in germplasm biostabilization.

Slow freezing. The first attempts at sperm cryopreservation were in fact K-VF [7], but at the rise of cryobiology this method did not ensure sufficient stability of the biological samples. Addition of a cryoprotectant glycerol essentially improved the results [2,4,18]. Glycerol and other permeable CPA allowed effective preservation of spermatozoa and then other cells, *e.g.* blood cells, at relatively low cooling rates. At the same time, long-term exposure to highly concentrated solutions kills the cells (the so-called "solute effect"). Freezing at rates surpassing the optimal rate of slow freezing (but insufficient for K-VF) leads to the formation of intracellular ice lethal for cells.

The two-factor hypothesis [15] explained the phenomenon of optimal rate of slow freezing, which promoted the development of cryopreservation techniques for different cell types, including animal sperm, oocytes, and embryos. However, slow freezing methods have two essential drawbacks. First, the optimal cooling rate depends on the cell type and more so on their size (more precisely, on the ratio of cell volume to surface area); it can differ by several orders of magnitude varying from several thousand degrees Celsius per minute for erythrocytes to fractions of degree Celsius for mammalian oocytes. Moreover, the optimal rate depends on membrane permeability for water (L_p) and CPA (P_{CPA}) and to a lesser extent on the type of the cryoprotectant *per se*. Thus, the optimal cooling rate for any new cell type should be determined in theoretical and experimental studies that can take a great deal of time. Second, specific combination of the optimal cooling rate and concentration of CPA is required to ensure maximum survival for each particular cell type. For instance, bone marrow stem cells demonstrate the best survival at cooling rate of ~2°C/min and CPA concentration of 1.25 M, while the maximum viability of bovine erythrocytes was observed at cooling rate of 1500°C/min and CPA concentration of 2.2 M [13]. Moreover, the most effective protocol of slow cryopreservation is an intricate multistep process [10]. It is specific for each particular cell type and requires expensive programmable freezers. The total cooling cycle can take long time (often more than 1 h).

Vitrification protocols: from E-VF to K-VF. Slow freezing showed its limitations for certain cell types (*e.g.*, oocytes). Vitrification of the whole rabbit kidney with the use of high concentrations of vitrificants and relatively low cooling rate [5] opened a new chapter in cryobiology. In fact, it was E-VF that can be called slow in comparison with more rapid K-VF. However, these rates considerably surpassed the optimal rates of slow cooling for the majority of cell types. In general, the optimal cooling rates for slow freezing vary from fractions of degree Celsius to hundred degrees Celsius per minute. For E-VF, cooling at a rate of several hundred and thousand degrees Celsius per minute is required, while K-VF totally free from exogenous vitrificants (with few exceptions, for example, sperm [7]) requires hyper-fast cooling (hundreds of thousands of degrees Celsius per minute).

Successful vitrification of mouse embryos with the use of the same high concentrations of vitrificants as for the kidney is also of paramount importance [19]. However, high concentrations of vitrificants (40-60% v/v). DMSO, ethylene glycol, propylene glycol, or glycerol are osmotically damaging and chemically toxic. They cannot be used for many cells, including oocytes and spermatozoa; the latter can withstand not more than 10-15% DMSO, ethylene glycol, propylene glycol, or glycerol. Therefore, E-VF is to be replaced with the much more rapid K-VF, especially for oocytes that are intolerant to slow freezing and E-VF mainly because of osmotic fragility of their cytoskeleton. Many modern cryopreservation protocols and cryocontainers were designed for vitrification of oocytes and embryos, but they all require small sample volumes and precise timing, which makes them vulnerable to technical errors.

Leidenfrost effect — influence on the maximum cooling rate during vitrification. It is generally assumed that the use of small vitrification cryocontainers (minicarriers) allows achieving relatively high (tens of thousands degrees Celsius per minute) cooling rates [6,9], but this assumption does not take into account the Leidenfrost effect. This effect describes the

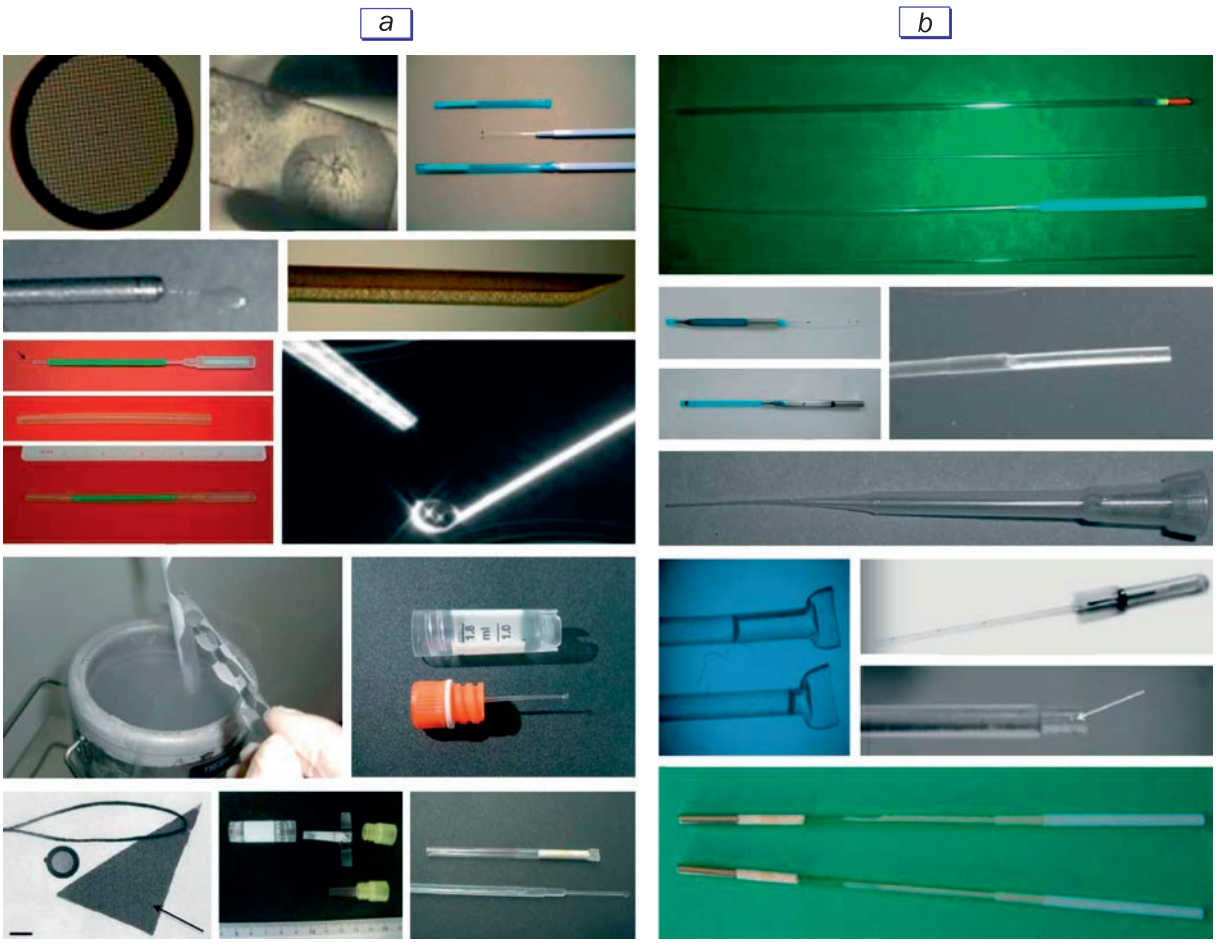

Fig. 1. Carrier systems used for vitrification of oocytes and embryos. *a*) Surface carriers; *b*) closed carrier [20].

formation of a vapor film that covers "hot" sample (compared to liquid nitrogen temperature LN_2, the initial difference of temperatures between the sample and coolant ΔT_e is 120°C and more). The formation of a vapor film, especially at temperatures close to Leidenfrost temperature, prevents heat transfer, because the heat flux from the sample surface during film boiling is by one to two orders of magnitude lower than during bubble boiling occurring at a lower ΔT_e (<30°C). For water, the ratio of maximum heat flux q_{max} (at ΔT_e=30°C) to minimum q_{min} in the Leidenfrost point (at ΔT_e=120°C) is ~50.

For liquid nitrogen LN_2, the ratio q_{max}/q_{min} (24) is somewhat lower, but lies in a narrower range of ΔT_e (from 14 to 35°C for q_{min} and q_{max}, respectively) [1]. This corresponds to 60-fold lower heat transfer coefficient ($h \equiv q/\Delta T_e$) in the Leidenfrost point. Taken together, these data indicate that cooling rates for small samples, and can be proportionally lower, cannot exceed 200,000-300,000°C/min for very small samples and be by one order of magnitude lower for samples with a volume >10 ml. At these cooling rates, relatively high and potentially dangerous concentrations of vitrificants (DMSO and ethylene glycol in concentrations ≥30%) are required.

Elimination of the Leidenfrost effect. The problem of increasing cooling rate can be radically solved via the development of techniques and instruments allowing complete elimination of the Leidenfrost effect. This device should be scalable, which would easily increase the volume of vitrified samples. The solution was found in spheres that are far from biotechnology: firefighting and rocket production, where cooling liquid is supplied to the cooled object under high pressure that allows quick and effective cooling of hot (or burning) surface. This forced convection significantly improves heat flux from the surface of the hot object (compared to the temperature of the coolant) and eliminates the Leidenfrost effect. The main difference of the proposed method of hyper-fast cooling is that the object is not plunged into the tank with liquid nitrogen, but is cooled with intense flow of cryogenic liquid supplied under high pressure.

***KrioBlast™-1*: a pilot (first) system for hyper-afast cooling.** The system for this method of cryogenic cooling was developed at CELLTRONIX [8].

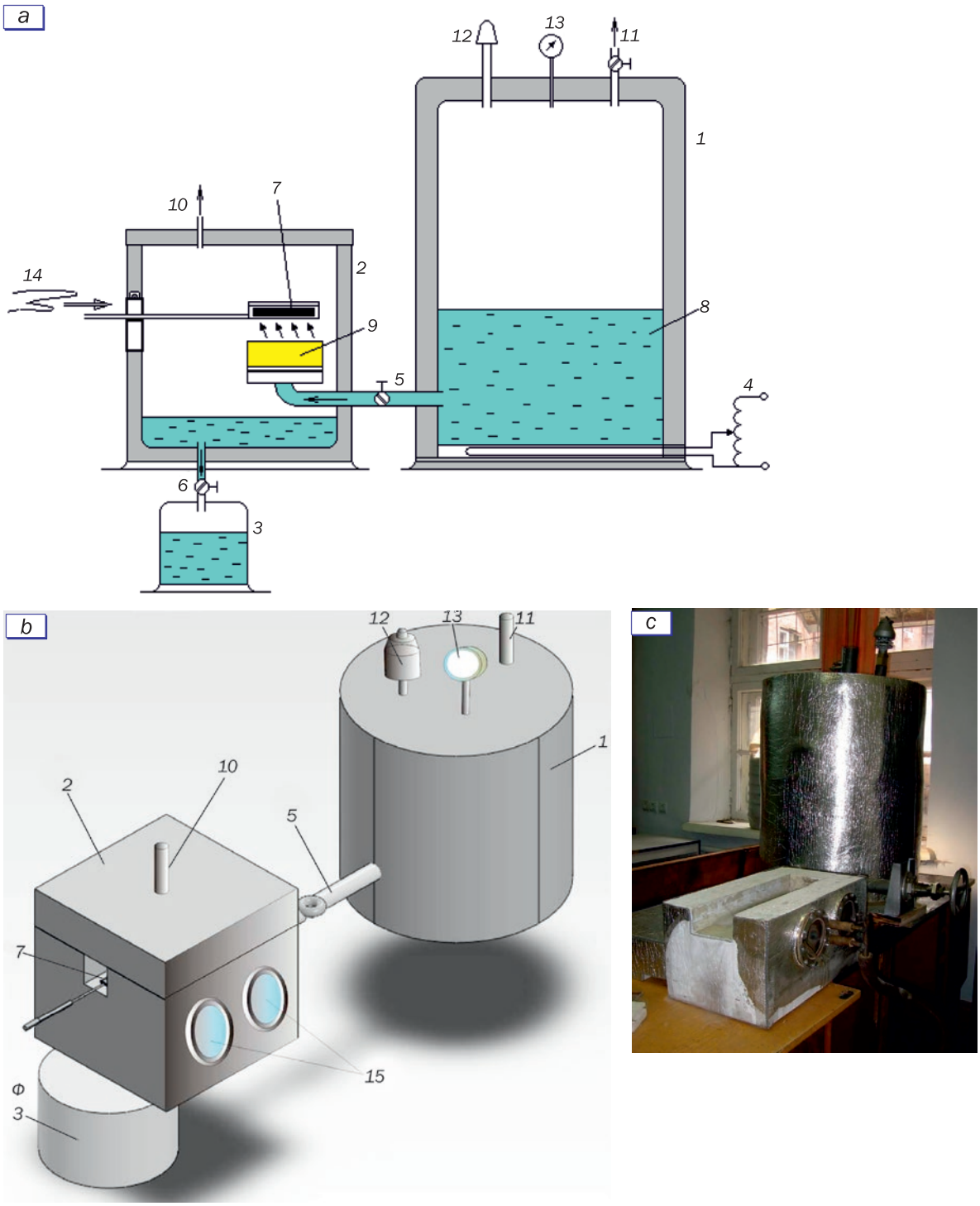

Fig. 2. *KrioBlast™-1*, prototype manually operated device for hyper-fast cooling (first generation). *a*) 2D scheme, *b*) 3D configuration, *c*) photograph of the system without upper cover of the cooling chamber. *1*) High-pressure vessel with liquid nitrogen (LN_2); *2*) cooling chamber; *3*) Dewar vessel for waste LN_2; *4*) adjustable heater for creation high LN_2 pressure; *5*) pipe with a valve for supplying LN_2 at high pressure; *6*) pipe with a valve for the release of waste LN_2; *7*) hand plunger with a holder for a biological sample in the plate; *8*) LN_2; *9*) nozzle generating a "cryogenic shower" with high-speed LN_2 jets; *10*) pipe for nitrogen vapor discharge from the cooling chamber at normal pressure (1 atm); *11*) manual valve for nitrogen vapor discharge from high pressure LN_2 tank; *12*) safety valve for dumping high pressure (>2 atm) in LN_2 vessel; *13*) pressure gauge; *14*) operator's hand; *15*) side view of two vacuum-tight windows: left window serves for illumination of the sample in LN_2 with a laser beam; the right window serves for monitoring verification of the biological sample in LN_2. After hyper-fast cooling with LN_2 jets, the sample can be plunged in LN_2 volume near the window. The transparency of the vitrified biological sample and milky color of the crystalline solution in the plate can be seen and evaluated by observing the process through the window. The vacuum between the two panes of windows *15* is created by means of a vacuum pump (not shown) and prevents condensation (fogging) on the outer (warm) pane of the window.

The first-generation *KrioBlast™-1* was created. A *KrioBlast-1*, the prototype instrument with manual control of sample introduction into the cooling zone was constructed (Fig. 2).

In *KrioBlast™-1*, the rate of cooling to cryogenic temperatures was determined using marker glycerol solutions with known cooling rate, as a function of vitrificant concentration. The maximum cooling rate was determined by the minimum glycerin concentration at which the sample remained vitrified (the method was described in detail in [21]). Indirect measurements are used because thermal inertia of the thermocouple at high cooling rates does not allow measuring cooling temperature, as the whole process ends in milliseconds, or even in microseconds.

In experiments, solutions with glycerol concentrations from 0 to 60% v/v with 5% intervals were used. During cooling, vitrification of the sample was observed through the right window (Fig. 2, *15*). Vitrification was monitored by sample transparency or crystallization (milky color, opacity). It should be noted that glycerol in this case was used as a vitrificant, a marker of the cooling rate, but this does not mean that it should be used in KVF of cells.

In experiments, vitrification of 4000 µl 15% glycerol was achieved, which corresponded to cooling rate 100,000-200,000°C/min [21]. The results of experiments in model solutions and on cells will be described in the next report.

This is the first publication on a scalable hyperfast cooling system to cryogenic temperatures in which KVF is realized. The proposed system is characterized by versatility and reliability, is easily operated, and can be automated in the future.

In preliminary experiments, high cooling rates were not achieved and vitrification of more diluted glycerol solutions was impossible for the following reasons:

- the sample was manually moved to the cooling zone (Fig. 2, *14*);
- liquid nitrogen LN_2 flow rate in the used cryogenic "Charcot's douche" cannot be high enough to completely eliminate the Leidenfrost effect;
- the plate with the sample was closed with cellophane plastic;
- in the future, the cell suspension should be covered and securely isolated from liquid nitrogen streams.

All these points are taken into account in the next generation of the *KrioBlast™-2* system and will be described in the next publication, which will also include experimental data on KVF of pluripotent stem cells and spermatozoa performed with this system.

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