COMBINED APPROACH TO THE DEVELOPMENT OF PROTOCOL FOR VITRIFICATION OF BULKY BIOLOGICAL OBJECTS

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ABSTRACT

Any means of cryopreservation generally relies upon vitrification, in which cells survive in glass between ice crystals. Proposals of a simultaneous vitrification of entire samples without the formation of ice even predate the discovery of the first cryoprotectants, in 1940s. However, the first practical implementation of vitrification (murine preimplantation embryos) was carried out only in 1985, by Gregory Fahy and William Rall. The present report provides a brief overview of different glass types resulting from liquids in a living system converting into the glassy state at low temperatures, as well as the potential benefits of vitrification for long-term large-sized sized biological objects preservation. We specify the main outstanding obstacles for the implementation of bulky biological objects vitrification, as well as ways proposed by various researchers to solve some of them.

One of those obstacles is the toxicity of extremely high cryoprotectant concentration (up to 60 % v/w) required for vitrification. That's why we turned our attention to xenon (Xe). The main prospective benefit of using Xe as a cryoprotectant is its absolute nontoxicity on tissue and cellular level, while even the most lowtoxic cryoprotectants (such as glycerol, ethylene glycol etc.) are potentially dangerous due to osmotic effects.

In order to assess the cryoprotective potential of Xe we have conducted two series of experiments – with yeast (S. cerevisidae) and mammalian (CHO-K1 and NIH-3T3) cell cultures in custom-built bronze miniature hyperbaric chambers.

Yeast survival in presence of Xe at all tested pressures appeared to be much better than in control experiments and approximately equal or slightly better than with 5% DMSO or glycerol; in experiments with joint application of Xe and DMSO or glycerol results have been even better. But in case of mammalian cells convincing results have been achieved only in experiments with the fastest cooling achievable.

Molecular dynamics simulations of Xe clathrate hydrate and ice crystal growth indicate vitrification of water inside cells under combined application of Xe and DMSO/glycerol as a possible explanation of the results observed (details are described in our report "Possible Mechanisms of Cryoprotective Effect of Xenon").

Based on this hypothesis we offer approaches to both cooling (combined use of traditional cryoprotectants and clathrate forming gases for vitrification, patent applied), and to the subsequent heating (uniform heating of complex biological objects by means of electric and magnetic fields' phased emitters, guided by MRI thermometry, patent pending – discussed in yet another corresponding report at this conference), providing the theoretical background for the successful cryopreservation of bulky biological objects. And eventually – up to individual organs and whole organisms.

In addition, we offer some suggestions for improving the efficiency of gas perfusion for chilling procedure, and application of frequency and width-pulse electromagnetic field modulation within the initial stages of cooling.

Before describing the essence of our propositions, it wouldn't be inappropriate to recall some matters of common cryobiological knowledge.

Any means of cryopreservation generally relies upon vitrification, in which cells survive in glass between ice crystals. Even under slow programmable freezing cells are surrounded by unfrozen liquid between growing ice crystals [Mazur 1984].

Vitrification is a process whereby fluid becomes a solid during cooling (more precisely, exhibits most of the properties of solids) without any substantial change in molecular arrangement or thermodynamic state variables. Hence, no crystallization with the consequent heat release and volume increase. Proposals of a simultaneous vitrification of entire samples without the formation of ice [Luyet 1937] even predate the discovery of the first cryoprotectants, [Polge, Smith et al. 1949] in 1940s.

However, the first practical implementation of vitrification (murine preimplantation embryos) was carried out only in 1985, by Gregory Fahy and William Rall [Rall, Fahy 1985].

The main condition for the onset of vitrification during cooling is an increase of liquid viscosity up to about 10^{13} Poise. On the molecular level it is expressed in loss of rotational and translational degrees of freedom, leaving only bond vibration within a fixed molecular structure, which leads to a decrease in the specific heat and thermal expansion coefficient [Wowk 2010].

Cryoprotectants lower the critical cooling rate (which is not less than 10^7 °C/min [Armitage 1991] for pure water) required for vitrification inversely proportional to their concentration within the cooling solution, reducing it up to quite available speeds.

Cryopreservation of individual cell suspensions requires nothing more than a slow programmable freezing (SPF), and the vitrification protocols for whimsical preimplantation embryos [Isachenko, Alabart et al. 2003] are even more easy to apply. However, when dealing with macroscopic objects, researchers are faced with a number of additional obstacles, namely [Fahy, Wowk et al, 2009; Mazur, 2010; Hopkins, Badeau et al, 2012]:

a. The toxicity of extremely high cryoprotectant concentration (up to 60% v/w) required for vitrification.
b. Uneven cryoprotectants distribution in organ parenchyma.
c. Irregularity of cooling (temperature gradients).
d. Insufficient cooling rate.
e. Cracking during storage below glass transition temperature (T_g).
f. Occurrence of nucleation sites at about-Tg temperatures.
g. Insufficiently slow and uneven warming with temperature gradients and local overheating.
h. Devitrification during warming due to insufficient heating rate with ice recrystallization.
i. Reperfusion syndrome at the end of rewarming.

Ice formation during warming happens faster than during cooling because ice nucleation occurs at lower temperatures than ice growth. This nucleation leads to extensive ice growth at warmer temperatures. That's why the ''critical warming rates" (minimum warming rates to avoid "devitrification", or significant ice formation during warming from a vitrified state) are typically two or more orders of magnitude greater than critical cooling rates [Hopkins, Badeau et al. 2012].

The same stringent and even contradictory conditions are imposed on storage temperature and transport of macroscopic samples, because masses of vitrifiable tissues larger than a few cubic centimeters almost invariably develop large-scale fractures. Storing them at liquid nitrogen temperature leads to cracking due to shear stress relaxation, while keeping them close to the glass transition temperature (down to 15 degrees) results in the formation of nanoscale ice crystals due to lateral diffusion of water molecules, which increases the critical warming rate upon subsequent heating.

A detailed review of the entire spectrum of research requires a separate report, and goes beyond the scope of this work. The more so because no major success has been achieved yet – excluding successful vitrification of blood vessels (1996), peripheral nerves, pancreatic islets and so on. Although speaking of nerves and blood vessels, we consider it to be rather a tissue engineering than cryobiology.

The most promising advances in this field were made by "21st Century Medicine", a company led by Gregory Fahy and Brian Wowk. In 2005 they have reported on a rabbit kidney that survived vitrification and subsequent transplantation with immediate contralateral nephrectomy, successfully functioning for 9 days [Fahy, Wowk et al., 2009]. However, due to the above-mentioned unresolved problems, this experiment remains anecdotal so far.

We know three possible ways of macroscopic biological objects accelerated warming: the dielectric warming [Wusteman, Robinson et al. 2004], vascular perfusion with inert fluids that remain

liquid at cryogenic temperatures [Federowicz, Harris et al. 1999], and gas perfusion [Schimmel, Wajcner et al. 1964; Bickis & Henderson 1966; Hamilton, Holst et al. 1973; Van Sickle & Jones 2014].

Dielectric warming results in very uneven heating.

Cooling solutions to cryogenic temperatures directly by cryoprotectants is impossible, due to progredient solute viscosity and peripheral vascular resistance elevation. As a result, the perfusion rate decreases. From a certain moment, instead of passing through the microvasculature, refrigerants begin to "shunt" through the major vascular arcades. Respectively the heat exchange is complementarily weakened and cooling becomes irregular. With a further increase viscosity perfusion either stops or ruptures in the parenchyma occur. Liquid perfluorocarbon-based coolants are also unable to solve the problem of sufficient cooling rate for the same reasons, albeit at lower temperatures.

Gaseous coolants, despite their negligible heat capacity, have two to three orders of magnitude lower viscosity, and freely pass through the vascular bed even at cryogenic temperatures. The most popular in this area is helium, due to its low condensing temperature and the highest diffusion mobility.

Of course, the gas perfusion has its own shortcomings:

- low heat capacity forces the use of high pressure to increase the heat transfer, which is fraught with barotrauma;
- gas embolism - particularly during rewarming, when the gaseous coolant is replaced by a liquid;
- «drying up» of endothelium and the adjacent tissue areas.

In view of the foregoing, for vitrification of isolated organs (and eventually up to intact organisms), suitable for transplantation after storage and rewarming, we plan the following:

1. Application of combined use of traditional cryoprotectants with clathrate forming gases as a means for vitrification. We turned our attention to xenon [Prehoda 1969; Rodin, Isangalin et al. 1984; Shcherbakov, Tel'pukhov et al. 2004; Sheleg, Hixon et al. 2008] because of its absolute non-toxicity on tissue and cellular level, while even the most low-toxic cryoprotectants (such as glycerol, ethylene glycol etc.) are potentially dangerous due to osmotic effects.

   In order to evaluate the cryoprotective potential of xenon we have conducted two series of experiments – with baker's yeast (S. cerevisiae) and mammalian cell cultures (CHO-K1 and NIH-3T3) in custom-built bronze miniature hyperbaric chambers.

   Yeast survival at all xenon pressures tested (3 to 7 at) after 2 hours of pressure chamber exposure at -20°C with subsequent chamber immersion in liquid nitrogen (68,5 plus or minus 6%) turned out to be better than in control (35 plus or minus 6%) and comparable or even higher to that of 5% DMSO or glycerol application (50 plus or minus 25%). Joint application of xenon + DMSO or glycerol showed 71 plus or minus 15% of cell survival.

   However, experiments with mammalian cells at xenon partial pressures of 2.5 to 12 at upon slow programmable freezing (SPF) showed complete cells dissolution upon conventional rewarming. Even after designing a sophisticated decompression protocol we could not achieve survival upon thawing. This made us disappointedly conclude that xenon has no cryoprotective properties applying SPF. Nevertheless, 2,8 plus or minus 2,3% of control group cells frozen by the abovementioned "yeast" protocol of cells survived and, moreover, fastest achievable cooling speed (direct placing of pressurized hyperbaric chamber into liquid nitrogen, which we considered an absolute death sentence) in the other control experiment improved cells survival up to 22,5 plus or minus 13,4%.

   Large scale molecular dynamics simulations of xenon clathrate hydrate and ice crystal growth (details are recently described in our report "Possible Mechanisms of Cryoprotective Effect of Xenon") indicate vitrification of water inside cells, especially under combined application of xenon and DMSO, as a possible explanation of these results.

The main thing for us is that the hydrophobic solubility of xenon in low temperature water increases with cooling faster than the thermal stability of clathrate hydrate [Artyukhov, Pulver et al. 2014].

As a result, it should turn out that traditional cryoprotectants, by suppressing xenon crystalline hydrate formation on cooling stage, would allow the solution to be saturated by xenon to a significant increase in liquid viscosity. This will enable vitrification using a much lesser amount of chemical cryoprotectants, and thus yield large decrease in toxicity. We have submitted a patent application.

Sounds so simple, even primitive. Why didn't anybody think of this before?..

2. Second critical step is to develop a methodology for a fundamentally new approach to a uniform heating of complex biological objects by means of electric and magnetic fields' phased emitters, guided by MRI thermometry – also patent pending and discussed in yet another corresponding report at this conference.
3. Creation of an improved carrier solution for cryoprotectants with an emphasis on organ conditioning, prevention of chilling injury, neutralization of reactive oxygen species, activation of apoptosis cascades and overall reperfusion syndrome during rewarming.
4. Slight improvement of gas perfusion methodology. Increasing the heat exchange efficiency should be achieved through a conjunction of elevated persufflation pressure with modulation of sonic and ultrasonic waves.
5. Application of frequency and width-pulse electromagnetic field modulation within the initial stages of cooling, not confining to a simple 50 Hertz oscillation used in Cells Alive System low-temperature freezer of Abiko corporation [Owada, Saito 2010].
6. Determination of the best storage temperature for objects, vitrified by our method of (taking into account the alleged stabilizing effect of gas cryoprotectants).

REFERENCES

1. Armitage W.J. Preservation of viable tissues for transplantation // B.J. Fuller, B.W.W. Grout (Eds.). Clinical Applications of Cryobiology / CRC Press, 1991, P. 169–190.
2. Artyukhov V.I., Pulver A.Yu., Peregudov A., Artyuhov I. Can xenon in water inhibit ice growth? Molecular dynamics of phase transitions in water-Xe system // J. Chem. Phys. 141, 034503. 2014, doi:10.1063/1.4887069.
3. Fahy G.M., Wowk B., et al. Physical and biological aspects of renal vitrification // Organogenesis, 2009, 5(3), P. 167–75.
4. Federowicz M.G., Harris S. B. et al. A method for rapid cooling and warming of biological materials // United States Patent 6274,303, 1999.
5. Bickis I.J., Henderson I.W.D. Helium Perfusion Technique Advances // JAMA, 1966, 197(11), P.36–37.
6. Hamilton R., Holst H.I. et al. Successful preservation of canine small intestine by freezing // J. Surg. Res., 1973, 14(4), P.313–8.
7. Hopkins J.B., Badeau R. et al. Effect of common cryoprotectants on critical warming rates and ice formation in aqueous solutions // Cryobiology, 2012, 65(3), P.169–178.
8. Isachenko V., Alabart J.L. et al. New technology for vitrification and field (microscope-free) thawing and transfer of the small ruminant embryos // Theriogenology, 2003, 59, P. 1209–1218.
9. Luyet B.J. Differential staining for living and dead cells // Science, 1937, 85(2195):106.
10. Mazur P. Freezing of living cells: mechanisms and implications // Am. J. Physiol., 1984, 247, P. C125–C142, 158.

11. Mazur P. A biologist's view of the relevance of thermodynamics and physical chemistry to cryobiology // Cryobiology, 2010, 60(1), P. 4–10.
12. Owada N., Saito S. Quick Freezing Apparatus and Quick Freezing Method // United States Patent 7810, 340 (2010).
13. Polge C., Smith A.U., Parkes A. S. Revival of spermatozoa after vitrification and dehydration at low temperatures // Nature, 1949, 164(4172):666.
14. Rall W.F., Fahy G.M. Ice-free cryopreservation of mouse embryos at -196 degrees C by vitrification // Nature, 1985, 313(6003):573-5.
15. Prehoda R.W. Suspended Animation: The Research Possibility that May Allow Man to Conquer the Limiting Chains of Time. Chilton Book Company, 1969.
16. Rodin V.V., Isangalin F.S. et al. Structure of protein solutions in a presence of xenon clathrate // Cryobiology & Cryo-Medicine. 1984, 14, P. 3–7.
17. Shcherbakov P.V., Tel'pukhov V.I. et al. Method for Organs and Tissues Cryoconservation In Situ. 2004.
18. Sheleg S., Hixon H. et al. Cardiac Mitochondrial Membrane Stability after Deep Hypothermia using a Xenon Clathrate Cryostasis Protocol – an Electron Microscopy Study // International Journal of Clinical and Experimental Pathology, 2008, 1(5), P. 440–447.
19. Schimmel H., Wajcner G. et al. Freezing of whole rat and dog kidney by perfusion of liquid nitrogen through the renal artery // Cryobiology. 1964, 1(2), P. 171–5.
20. Van Sickle S., Jones T. Method and apparatus for prevention of thermo-mechanical fracturing in vitrified tissue using rapid cooling and warming by persufflation // United States Patent 5952,168, 2014.
21. Wowk B. Thermodynamic aspects of vitrification // Cryobiology, 2010, 60, P. 11–22.
22. Wusteman M., Robinson M., Pegg D. Vitrification of large tissues with dielectric warming: biological problems and some approaches to their solution // Cryobiology, 2004, 48, P. 179–89.