[4] Fahy GM et al.. Cryopreservation of organs by vitrification:perspectives and recent advances. Cryobiology 2004;48(2):157–78.

doi:10.1016/j.cryobiol.2010.10.030

Biophysics of cryobiology

27. Progress of vitrification: From single cell to multiple objects. L.L. Kuleshova *[1], S.S. Gouk[1], R. Magalhaes[1], F. Wen[1], Y.N. Wu[2], G.S. Dawe[3,4], H. Yu[5,6], [1] Low Temperature Preservation Unit, National University Medical Institutes, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, [2] Department of Orthopaedic Surgery, NUS Tissue Engineering Program, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, [3] Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, [4] Neurobiology and Ageing, Centre for Life Sciences, National University of Singapore, Singapore, [5] Department of Physiology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore, [6] Institute of Bioengineering and Nanotechnology, Agency for Science, Technology and Research, Singapore

Vitrification achieves cryopreservation without the formation of ice crystals, and therefore benefits cells and tissues known to be vulnerable to ice damage during conventional freezing. It is widely reported that oocytes, stem cells and other sensitive cells are adversely affected by freezing. Vitrification offers an improvement in both survival and non-lethal cell injury. Several areas of life sciences can significantly benefit from development of vitrification strategy. Vitrification of single object is now very well understood. My success on development of vitrification strategy leading to the world-first live birth from a vitrified oocyte is the last step in the line. This was accomplished 100 years after vitrification as a concept was proposed in physics, namely in 1898. Advancement in the area leads to development of vitrification strategies for multiple objects.

We developed vitrification of multiple objects such as neuronal stem cells in their spherical form and more complex tissue engineered constructs involving hepatocytes for urgent metabolic support during liver failure. The formulation of improved vitrification solutions is an integral part in developing this vitrification strategy. To achieve this goal, microencapsulated hepatocytes and hepatocyte spheroids were used as the model systems. In regards to implementation of this new cryopreservation strategy in clinical practice, we were the first to originate the idea that serum/proteins are not essential during vitrification, and published successful results which eliminating their usage; this is significant because addition of serum and proteins may induce differentiation during passaging or compromise the sterility of stem cells if they are used for therapeutic transplantation. We also proved efficacy of this strategy for a platform technology involving human adult stem cells. As we have achieved vitrification of large number of neurospheres sufficient for human treatment, we envision feasibility of vitrification of mesenchymal stem cells in large quantities. We reported a way to achieve effective preservation of large constructs involving primary cells used as a part of bioartificial liver device. Cryobiology of tissue-engineered complexes has now been developed in my laboratory, and this research will provide a vital therapeutic opportunity.

We attempted to confirm that vitrification has an advantage of causing less mechanical stress to the biological material over freezing on cellular level. In a recent study we observed the actin underlining of the cellular membrane and also the actin distribution through the cytoskeleton, two of the sub-cellular arrangements that would be greatly affected by mechanical damage. Our study on the effect of different cryopreservation strategies on the cell actin distribution and membrane thinning (or most specifically the thinning of the actin band underlying the cellular membrane) clearly showed that the detrimental effects of cryopreservation by freezing are much more prominent than those incurred by the hepatocytes preserved using the vitrification approach. Therefore, we may conclude that vitrification as the concept of cryopreservation has advanced from single non-sensitive cells to the sensitive cell (e.g. human egg) and currently handling of multiple biological objects, even in up-scaled conditions. There is no doubt in universality of vitrification strategy.

Conflict of interest: None declared.

Source of funding: None declared.

doi:10.1016/j.cryobiol.2010.10.031

28. Fast measurement of cooling and heating rates in standard cryopreservation protocols based on vitrification. Aline Schneider Teixeira *[1], Marha Elena Gonzalez-Benito[2], Antonio Diego Molina-Garcha[1], [1] Instituto del Frío-ICTAN (CSIC), José Antonio Novais 10, 28040 Madrid, Spain, [2] Dept. Biología Vegetal, Universidad Politécnica de Madrid, Ciudad Universitaria, 28040 Madrid, Spain

Some cryopreservation protocols rely on fast transfer from liquid to glassy state, avoiding the formation of intracellular ice crystals. Ice can be equally formed during cooling and warming processes. Protocols include additives and steps designed to reduce freezing equilibrium (T_f) and increase glass transition (T_g) temperatures, as well as to confer specific protection to cells against several associated stresses generated during the cryopreservation process (cold pre-treatment, dehydration and oxidative stress). These additives and pre-treatments also contribute to reduce the probability of ice nucleation at all temperatures, mainly through micro-viscosity increase. Still, there is a risk of ice formation in the temperature region comprised between $T_f$and T_g. Consequently, fast cooling and warming, especially in this region, is a must to avoid ice-derived damage.

In spite of the importance of temperature change rates, they have been scarcely experimentally measured. Temperature evolution of samples simulating real cryopreservation protocols has been measured in this work and the results are comparatively presented.

A very fast temperature data acquisition system was employed (50 measurements per second), producing enough data points in the interval of interest (considered here from 0 °C to −150 °C), even in the fastest cases (1–2 s). The precision of the experimental measurement system employed was evaluated and a good reproducibility was found among repeats, in spite of the experimental variation inherent to custom cryopreservation practice. Several techniques based in fast cooling published in the literature (vitrification, encapsulation-dehydration, droplet) were studied, together with the described warming procedures. A large difference in temperature change rates can be found among methods, ascribed to differences in mass, heat capacity and thermal conductivity of samples and barriers (containers). Differences between cooling and warming rates in the same experimental set were smaller and, for most cases, suggested no additional ice formation risk during the warming process. In some cases in which protocols included a definite warming time period in a thermostatic bath, it was observed that this time elapsed before the sample temperature had risen from 0 °C.

The probability of ice formation under $T_f$and over T_g always exits. Although pretreatments can largely limit this probability, both by molecular mobility and freezable water content reduction, they may also cause decreases in viability, due to dehydration or cytotoxic derived damages, and produce alterations in the recovered material, also derived from too drastic stress conditions. A reduction in these stresses can be made compatible with successful cryopreservation by increasing cooling and warming rates.

Conflict of interest: None declared.

Source of funding: This work was funded from project "*CRIOFIS*" (AGL2007-65938-C02-02/AGR) from the Spanish "*Plan Nacional de Investigaciyn CientHfica, Desarrollo e Innovaciyn Tecnolygica 2004-2007*", *M.E.C.*.

doi:10.1016/j.cryobiol.2010.10.032

29. Identification of supercooling-facilitating (anti-ice nucleating) hydrolyzable tannins from xylem parenchyma cells in katsura tree (Cercidiphyllum japonicum). Donghui Wang *[1], Jun Kasuga[2], Chikako Kuwabara[1], Yukiharu Fukushi[1], Keita Arakawa[1], Seizo Fujikawa[1], [1] Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan, [2] Cryobiofrontier Research Center, Faculty of Agriculture, Iwate University, Morioka 020-8550, Japan

Xylem parenchyma cells of katsura tree exhibit deep supercooling capability to −40 °C. Our previous studies showed that diverse kinds of supercooling-facilitating (anti-ice nucleating) substances might exist in these xylem parenchyma cells, although we have currently identified only four kinds of supercooling-facilitating flavonol glycosides in the xylem parenchyma cells of katsura tree (Kasuga et al., Plant Cell Environ. 31 (2008) 1335–1348). In this study, therefore, we tried to identify other types of supercooling-facilitating substances from xylem parenchyma cells in katsura tree.

Four to six-year-old twigs from an adult katsura tree (*C. japonicum*) grown in an experimental field of Hokkaido University were used for isolation of new compounds with supercooling-facilitating capability. Crude xylem extracts were extracted with methanol from xylem and they were separated by liquid–liquid extraction using ethyl acetate (EtOAc). The resultant EtOAc fraction was further separated to about 20 fractions by silica gel column chromatography. All these silica gel fractions exhibited supercooling-facilitating capability, and our previous study showed the existence of flavonol glycosides in the silica gel fraction with the highest supercooling capability (Kasuga et al., ibid). In this study, we used different silica gel fractions that also exhibited high supercooling capability in order to identify different types of supercooling-facilitating substances. One silica gel fraction, which exhibited high supercooling capability in second order among all silica gel fractions, was separated by Sephadex LH-20 column chromatography and then subjected to HPLC analysis. Finally, chemical structures of compounds in three peaks of HPLC, which exhibited high supercooling capability, were analyzed by using a MS and NMR.

From deep supercooling xylem parenchyma cells in katsura tree, we identified three kinds of supercooling-facilitating hydrolyzable tannins. These were 2,3,6-tri-*O*-galloyl-α,β-D-hamamelose (Kurigalin), 1,2,3,6-tetra-*O*-galloyl-*β*-D-glucopyranose, and 1,2,6-tri-*O*-galloyl-*β*-D-glucopyranose. Our results suggested that the presence of diverse kinds of supercooling-facilitating substances, including previously identified flavonol glycosides and hydrolyzable tannins, in xylem parenchyma cells produced high supercooling capability in xylem parenchyma cells of katsura tree.