Efficient Automatable and Aseptic Vitrification of Human Pluripotent Stem Cells Using Bio-Safe and Chemically Defined Media

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1. OBJECTIVE

Human pluripotent stem (hPS) cells, mainly represented by human embryonic stem (hES) and human induced pluripotent stem (hiPS) cells, are considered as a virtually unlimited material for biomedical research and cell-based therapies. However, their use in the clinics requires efficient and bio-safe handling. Cryopreservation is an obligate key step of storage and transportation, during which the cells undergo extreme physical and chemical conditions prone to alter their viability as well as their biological properties. Human PS cells cryopreservation routinely consists in conventional slow-freezing resulting in poor survival rates mainly due to cell damages due to excessive dehydration and water crystallization. In order to prevent this, several groups have adapted vitrification protocols to hPS cells. Vitrification is a cryopreservation method based on the conversion of a liquid into a glass-like state by an infinite enhancement of its viscosity and without formation of ice crystals. It has been shown that vitrification protocols are more effective than slow freezing (1–3). However, they cannot provide any insurance of biological safety since cells are stored in containers that are predisposed to leakage when plunged into liquid nitrogen (i.e. non aseptic containers). This study presents our newly developed hPS cells cryopreservation method.

2. MATERIALS AND METHODS

Our hPS cells (RCM-1 [hES cells], gRips and dKips [hiPS cells]) cryopreservation method is based on aseptic and automatable vitrification in sealed french straws. Only bio-safe and chemically defined cryopreservation media are used. To assess its efficiency, our method has been compared to conventional slow freezing and tested for post-warming recovery rates, morphology, growth and differentiation. Maintenance of pluripotency and cytogenetic stability were also evaluated.

3. RESULTS

To compare aseptic vitrification and conventional slow-freezing, we have estimated the recovery rates 24h after warming by a necrosis/apoptosis assay (Figure 1) and maintenance of undifferentiated status by morphometric analyses (Figure 2). Our aseptic vitrification method is more efficient than conventional slow freezing in terms of cell survival and maintenance of undifferentiated status. Concerning cell morphology, no obvious difference can be really underscored.
Figure 1: Assessment of hiPS cells survival rate (dKips) 24h after warming by apoptosis assay (FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen)) (VIT: n=8; SLF: n=4).

Figure 2: Assessment of maintenance of undifferentiated status of hES cells by morphometric analyses: ratio undifferentiated cells occupancy areas/total cells occupancy areas. Analyses were performed before cell culture reached confluence (VIT: n=22; SLF: n=19).

To confirm the maintenance of pluripotency status after aseptic vitrification, the expression of several markers was examined along time and for up to ten passages after warming. Immunofluorescent staining showed that hPS cells expressed the pluripotency transcription factors Oct4 and Sox2 as well as the cell surface markers TRA-1-81 and SSEA-4.
after aseptic vitrification. These results were confirmed and quantified by FACS analyses. A strong phosphatase alkaline activity was also detected after warming of vitrified hES cells. The pluripotency status of vitrified hPS cells was also assessed in vivo after warming. After their injection into dorsal flanks of immunodeficient mice, hPS cells produced teratomas at the injection site. Their analysis allowed the identification of tissues stemming from the three germ layers. Finally, the cytogenetic stability of RCM1 cells was carried out by karyotyping. Before and after vitrification, a normal 46,XX karyotype was observed in the majority of spreads. These different assays shown that, after our aseptic vitrification procedure, hPS cells keep their biological and cytogenetic properties after warming.

4. CONCLUSION

We have demonstrated on hPS cells that, despite additional constraints (aseptic, defined medium, automatable…), our aseptic vitrification method is more efficient than conventional slow freezing. We can also conclude that the cells keep their biological (pluripotency) properties after aseptic vitrification. This higher efficiency can be explained by the conjunction of short exposure periods to cryoprotectant solutions and high cooling and warming speeds that leads to lower intracellular concentration of cryoprotectants after vitrification than after conventional slow freezing (4).

Aseptic vitrification of hPS cells is now possible in completely defined media. Because straws are directly immersed in liquid nitrogen, this method does not require any specific and expensive material. To limit cell manipulations, our protocol implies stepwise addition and dilution of cryoprotectants before cooling and after warming, respectively, allowing automation. Our automatable and aseptic vitrification technique making use of bio-safe and chemically defined media is, to our knowledge, the first method combining all these properties.

5. POTENTIAL APPLICATIONS

Our technique appears as very promising for the storage and transportation of hPS cells or other cells, particularly when intended to clinical applications that require high standards of efficiency, safety and traceability.

6. POTENTIAL FOR VALORIZATION

The rapidly expanding field of stem cells-based research and clinical applications is a highly favourable context for the implementation of novel methods allowing efficient, user-friendly and automatable cryopreservation, still addressing safety and regulatory constraints. Furthermore, our method has been validated on various “sensitive” stem cell-like lines and embryos from human and non-human species. The adequation of our results to the abovementioned context is very encouraging regarding the valorization potential. This work should be valorized thanks to a Wallonia First Spin-Off grant (Vitriscell).

References

