Cryopreservation of Adherent Cells: Strategies to Improve Post-Thawing Viability and Function

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Introduction

Clinical and commercial availability of cell-based products for tissue engineering and regenerative medicine require effective methods for their long-term storage in cryobanks, which are not yet established for complex systems such as cell monolayers, tissues or biosynthetic constructs [1].

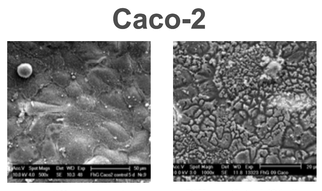
Cell entrapment in a gel is a promising cryopreservation strategy to improve post-thaw viability and function of cell types which were shown to poorly survive the cryopreservation process [2,3].

In this work, combined strategies for the cryopreservation of adherent cells were investigated based on cell entrapment in clinical-grade, highly purified alginate of extremely high viscosity (0.1% w/v viscosity in distilled water > 30 mPa.s) uniformly cross-linked with Ba^{2+} [4].

As model systems, Neuroblastoma N2a and Caco-2 Colon Adenocarcinoma cell lines were used due to their specific characteristics, which makes them interesting lines for studying the cryopreservation of differentiated cells [5]. As the cryopreservation medium, serum-free CryoStor™ (Biolife Solutions®) solution was compared with culture medium supplemented with bovine serum, both containing 10% Me_2SO.

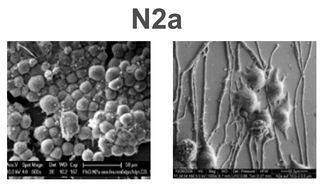
Cell Models:

Caco-2



Undifferentiated | Differentiated (21 d)

N2a



Undifferentiated | Differentiated (5 d)

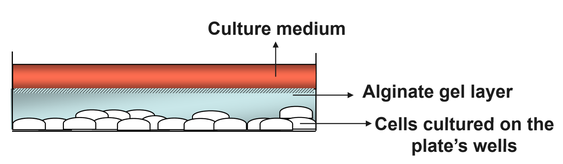
Aim and Strategy

Develop optimized methodologies for the cryopreservation of functional cell monolayers for cell-based therapies and *in-vitro* pharmacological studies

- Improve cell viability
- Improve cell-specific function
- Avoid monolayer's detachment
- Avoid lost of cell-cell contact

STRATEGY

Monolayer's entrapment beneath a layer of ultra-high viscous (UHV) alginate



Methods

Culture: Caco-2 and N2a cells were cultured on 4-well plates in either a non-differentiated or fully differentiated state. Caco-2 cells spontaneous differentiation into enterocyte-like cells was achieved through long-time culture. Neuronal differentiation of N2a cells was induced through retinoic acid addition to low-serum content medium. After 1 or 4 days post-inoculation, a thin layer of UHV alginate cross-linked by Ba^{2+} ions was added over the cells on the plates.

Cryopreservation: After 5 days of culture (or 21 days for differentiated Caco-2 cells), cells were frozen at 1°C/min to -80°C inside the plates with either serum-supplemented culture medium or CryoStor™-CS10 (BioLife Solutions, Bothell, WA, USA), both containing 10% Me_2SO, and stored at -80ºC during at least 1 week.

Post-thawing characterization: Cell viability was assessed through a membrane integrity assay and the metabolic assay alamarBlue™. The structural integrity and differentiation state of the cells was evaluated through scanning electron microscopy. Maintenance of cell differentiated state after thawing was assessed through biochemical and immunohistochemical assays, respectively.

Evaluated parameters:

Differentiation state	Undifferentiated	*Versus*	Differentiated
Time of alginate addition over the cells	1 day post-inoculation	*Versus*	4 days post-inoculation
Cryopreservation medium	Culture medium containing serum + 10% Me_2SO	*Versus*	Serum-free preservation solution CryoStor™-CS10

Results

Day 0 — Cell seeding on the surface of 4-well plates

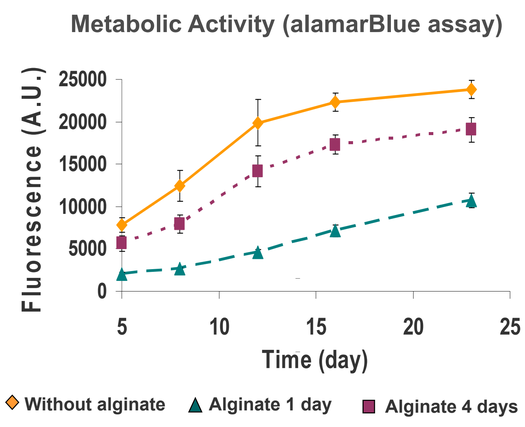
Day 1 or 4 — Alginate addition over the cells

Day 5 — Assessment of cell viability and differentiation state + Cell freezing on 4-well plates and storage at -80ºC

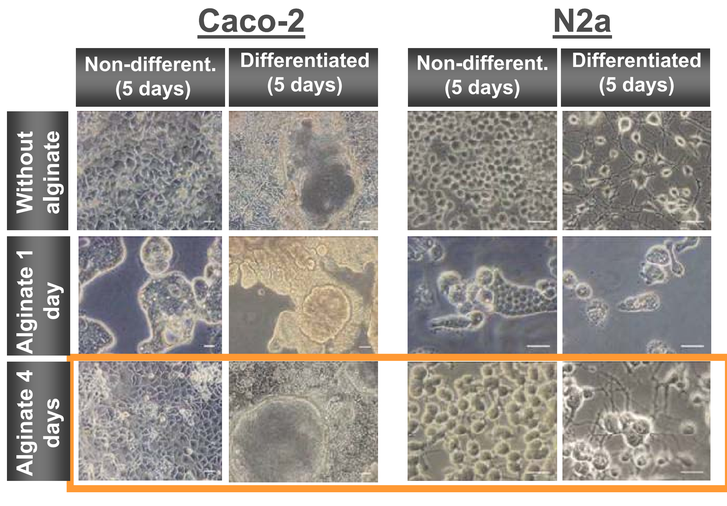
Day 12 — Thawing + Assessment of cell viability and differentiation state/capacity

Effect of alginate entrapment on cell growth and differentiation

Caco-2



Caco-2



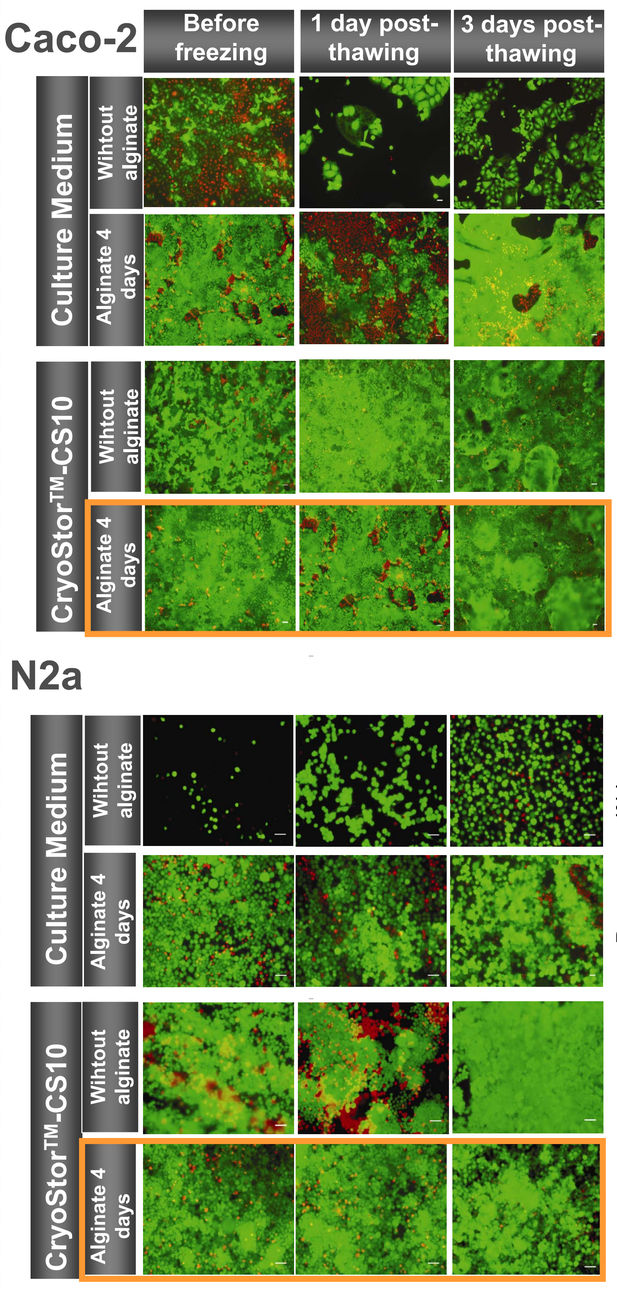
N2a

Alginate entrapment 4 days post-inoculation:

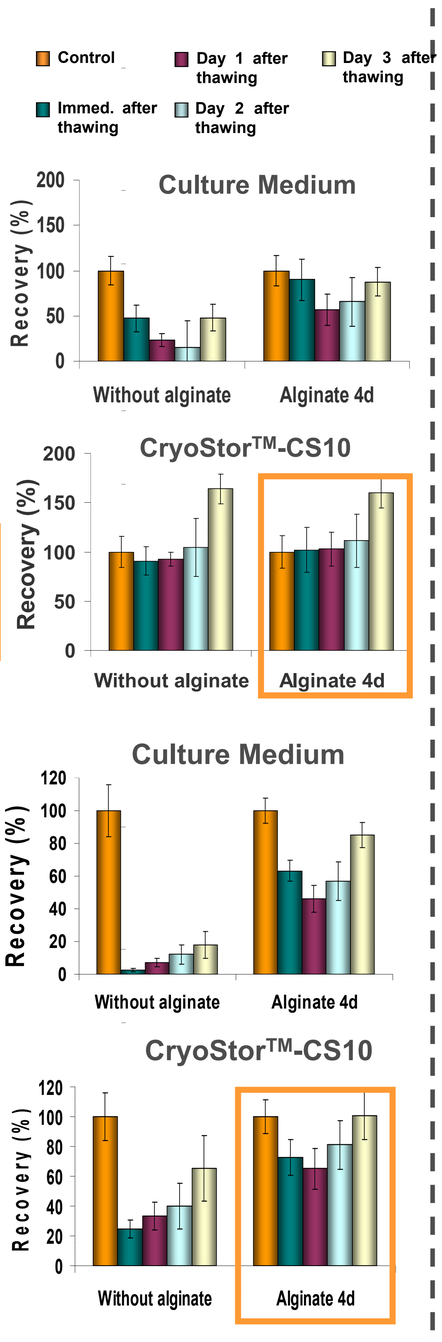
- ✓ Caco-2 cells proliferation rate and metabolic activity are not affected;
- ✓ Cell morphology is not affected;
- ✓ Cell differentiation state/ capacity is not affected;

Post-thaw recovery of non-differentiated monolayers

Membrane integrity:



Metabolic Activity:



✓ **Alginate entrapment** improves recovery of **culture medium** cryopreserved cells by minimizing membrane damage and cell detachment after thawing.

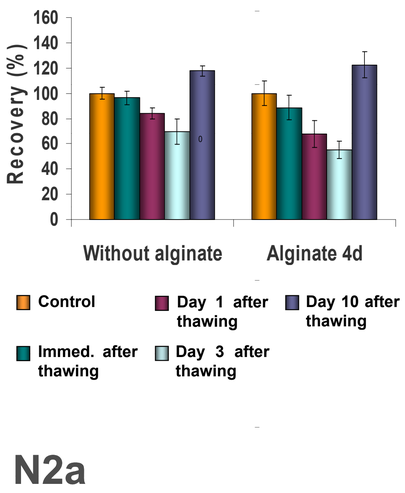
Nevertheless…Up to 50% death within 24 hours after thawing!

✓ **CryoStor™-CS10 solution** allows full recovery of metabolic activity and initiation of proliferation within 24 hours post-thawing.

Post-thaw viability and differentiation state of differentiated monolayers

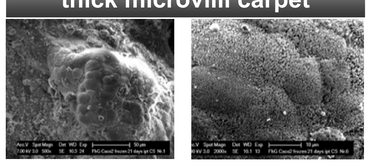
Caco-2

Metabolic Activity:

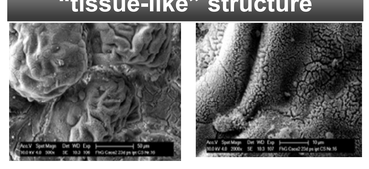


Scanning electron microscopy:

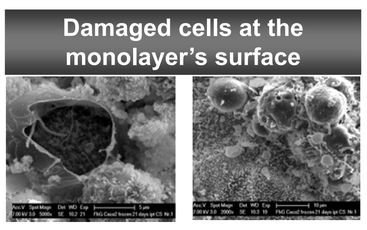
Undamaged cell surface with thick microvilli carpet



Shrinkage of the whole "tissue-like" structure



Damaged cells at the monolayer's surface

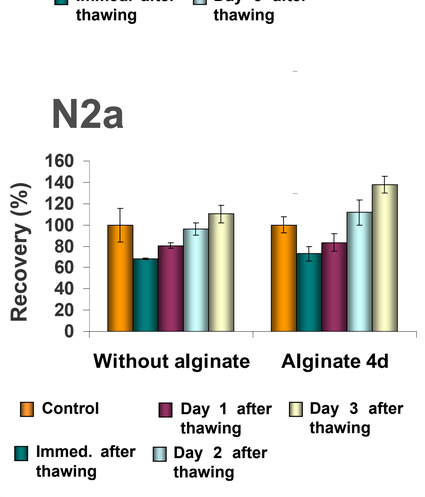


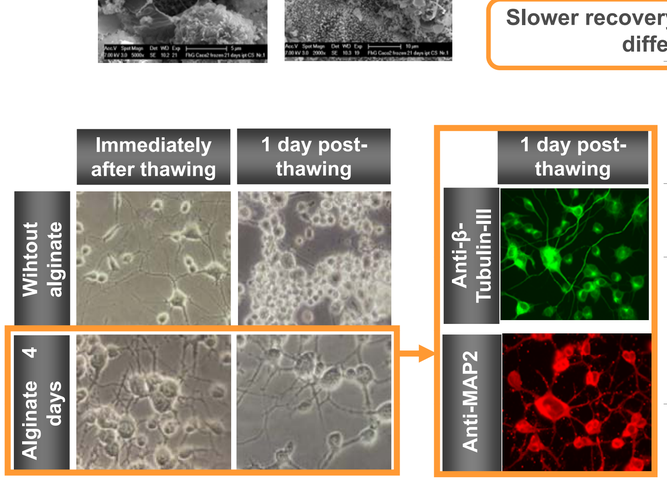
Multiple cell layers

Additional factors related to the three dimensional arrangement

Slower recovery when compared to non-differentiated cells

N2a





Alginate entrapment:

- ✓ Maintenance of neuronal networks integrity;
- ✓ Expression of typical neuronal markers.

CONCLUSIONS

- Monolayer entrapment beneath an alginate layer improves cell recovery by avoiding detachment from the surface and breakage of cell-cell interactions.
- The use of CryoStor™ solution improves the cryopreservation process for both cells lines, allowing the maintenance of high post-thaw recovery of viability and differentiation state.

An efficient novel strategy for successful cryopreservation of ready-to-use cell monolayers was validated based on cell entrapment in clinical grade, UHV alginate and the use of CryoStor™ solution

✓ **Supports the implementation of routine cryopreservation practices for engineered cells and tissues and their immediate availability for cell-based therapies.**

References

[1] Baust, J. G. and Baust, J. M., Advances in Biopreservation CRC Press:63-87(2006).
[2] Mahler, S. M. *et al.*, Cell Transplant. 12 (6): 579-92 (2003).
[3] Inaba, K. *et al.,* Transplantation 61 (2): 175-9 (1996).
[4] Zimmermann, H. *et al.*, J. Mat. Sci.: Mat. Med. 16(6): 491 – 501 (2005).
[5] Malpique, R. *et al.*, Biotechnol. Bioeng. 98(1): 155-66 (2007).

Acknowledgments

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