Cryopreservation of Human Precision-cut Lung Slices Provides an Immune Competent, Pulmonary Test System for "On-demand" Use

Holger Behrsing[1], Méry Marimoutou[1], Khalid Amin[2], Andreas Stucki[3], Amy J. Clippinger[3], Adam Wahab[1], Lindsey Ukishima[1], Vivek S. Patel[1]

[1]Institute for In Vitro Sciences, Inc., Gaithersburg, Maryland, USA [2] Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN, USA [3] PETA Science Consortium International, Stuttgart, Germany

ABSTRACT

Human precision-cut lung slices (hPCLS) are considered a highly relevant 3-dimensional model of the lung. They offer native architecture and cells of the lung tissue including respiratory parenchyma, small airways, and immune competent cells involved in inflammatory and sensitization processes. Laboratories report long term hPCLS cultures (≥4 weeks) and results that reflect key events in lung disease progression in relevant adverse outcome pathways (AOP). However, a primary deficit of this test system is the availability of human donor lungs for research and the limited testing one can complete with a single donor's tissue.

Using refined methodology, modified tissue slicers, and a novel cryo buffer specifically designed to preserve hPCLS in a frozen state, these improvements (under optimal conditions) provide >1000 hPCLS from a single donor (healthy or diseased tissue) that can be stored long term. We compared one donor's fresh and cryopreserved (~7 and ~34 weeks) hPCLS in four week cultures to ascertain the impact of short- and long-term cryopreservation on performance. At 7, 14, 21, and 28 days groups (n=6) were maintained in medium or challenged with 0.1% Triton X-100 (TX-100) or 5 µg/mL lipopolysaccharide (LPS) for 24 hr before harvest. hPCLS were assayed for WST-8 viability and groups split (n=3 each) for lysis for protein content analysis or fixation for H&E and IHC (CD86, Aquaporin and Prosurfactant) histology evaluation. Collected media were evaluated for LDH leakage measurements and multiplex cytokine analysis.

Results indicate both fresh and cryopreserved hPCLS retain equivalent biomass (209 ± 63 and 191 ± 57 µg protein/slice, respectively), viability (1.1 ± 0.2 and 1.0 ± 0.3 OD_{450}/slice, respectively), response to the irritant Tx-100 (90 ± 14 and 94 ± 9 % total LDH, respectively), and retain robust cytokine responses (up to 30-fold LPS induction) over the 4 week period. Histology assessment showed well retained cytomorphology over 28-days, using both fresh and cryopreserved hPCLS.

The scarcity of human lung tissue available for research and the inability to conduct larger scale testing when needed has limited the use of hPCLS as a test system for routine, high-throughput testing. With improvements in slice creation, storage, and culture conditions the hPCLS can be applied for larger scale testing, tissue banking, and repeat donor experimentation while retaining tissue integrity and functionality. These improvements position hPCLS as an accessible, human-relevant, pulmonary test system suitable for testing inhaled materials, evaluating key events in AOP, and the evaluation of therapeutics "on-demand".

INTRODUCTION

hPCLS offer native lung architecture including small airways and respiratory parenchyma, making it one of the most physiologically-relevant non-animal models of the lower lung. These substructures within the hPCLS reflect the presence of the many cell types in the human lung, not present in other non-animal models used for toxicity studies. The complement of these cell types in hPCLS allows for a more realistic interpretation of complex pulmonary responses to various exposures. Reports of multi-week culture longevity and complex responses has positioned hPCLS as a candidate model to evaluate key events associated with severe lung disease.

However, with infrequent donor tissue availability and lack of reliable hPCLS preservation techniques, hPCLS are difficult to employ in research studies that require repetition. Moreover, excess hPCLS generated must be utilized immediately or discarded. Clearly, reliable preservation techniques coupled with optimized long-term culture method would position hPCLS as a pulmonary test system with increased utility and accessibility for researchers.

Here, we evaluated the performance of short- and long-term cryopreserved hPCLS for a period of 4 weeks in culture. The responses of the cryopreserved hPCLS to cytotoxic and inflammatory stimuli were evaluated in comparison to that of the fresh hPCLS from the same donor lung. The initial results suggest that researchers may be able to bank frozen hPCLS and return to the same donor tissue on multiple occasions.

METHODS

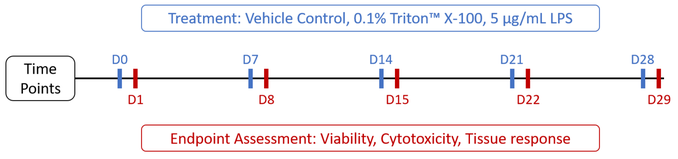

Figure 1. Treatment and endpoint assessment. The fresh or cryopreserved [~7 weeks (Cryo 1) or ~34 weeks (Cryo 2)] slices were acclimated at standard culture conditions for up to 4 days and then maintained in culture (DMEM/F12 medium) at air-liquid interface for ~4 weeks. A subset (n=6 per group) of hPCLS were treated with vehicle (VC), 0.1% Triton™ X-100 (TX-100) or 5 µg/mL Lipopolysaccharide (LPS) on days 0, 7, 14, 21, and 28. At 24 hours post-treatment (days 1, 8, 15, 22, and 29, respectively), the hPCLS were assessed for viability and then either lysed (n=3 per group) or fixed (n=3 per group) for biomass or histological evaluation, respectively. The media samples were used to measure released markers to assess cytotoxicity (LDH) and immune responses (IL-6, IL-8, MCP-1, GRO-α, and TIMP-1).

Generation of hPCLS and Cryopreservation: Upon receipt, the human lung was inflated using 0.8% agarose solution. Upon gelling, the lung was cut into ~1.5 cm thick sections and cylindrical cores were generated using 8 mm circular knife. The cores were sliced using a Krumkieck slicer with approximate slice thickness of 500 μm. Some of the fresh hPCLS were used for the experiments and remaining were cryopreserved using a cryo buffer developed by IIVS for storage of hPCLS.

Viability assessment: The viability of hPCLS was assessed using WST-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] assay.

Histological assessment: The formalin-fixed hPCLS sections were stained using H&E solutions and assessed for cytomorphology. The slices were scored for viability and overall health compared to the negative control tissues (N.C.; never-frozen hPCLS).

Multiplex analyte analysis: The levels of various analytes (IL-6, IL-8, MCP-1, TIMP-1, GRO-α) in the culture medium were determined using a Luminex assay kit (run on Luminex MAGPIX analyzer).

hPCLS Biochemical Characterization

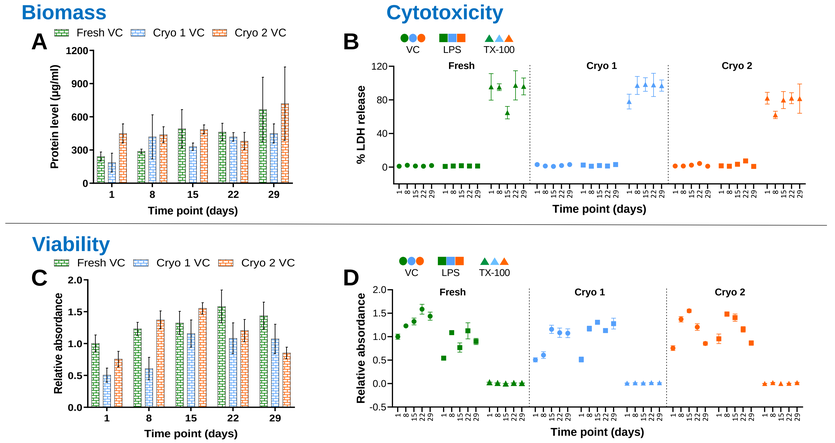

Figure 2. Characterization of cryopreserved hPCLS. The cryopreserved hPCLS were assessed for biomass, viability and responsiveness to stimulants like 0.1% TX-100 and 5 µg/mL LPS throughout the 4 week culture period (see **Figure 1** for time points). The hPCLS retained biomass **(A)** and viability **(C)**; OD450 values from WST-8 assay**)** during the culture period. The hPCLS were also responsive to the irritant TX-100 as indicated by increased cytotoxicity **(B)**; LDH release presented as percent of vehicle control group**)** and reduced viability **(D)**. N = 6 per group per time point.

hPCLS Immune Responses

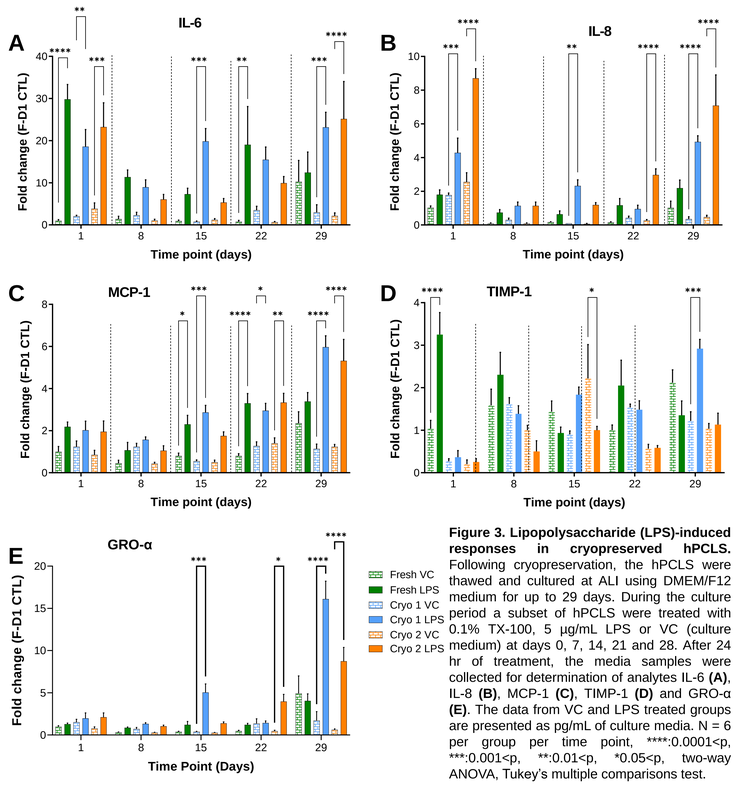

Figure 3. Lipopolysaccharide (LPS)-induced responses in cryopreserved hPCLS. Following cryopreservation, the hPCLS were thawed and cultured at ALI using DMEM/F12 medium for up to 29 days. During the culture period a subset of hPCLS were treated with 0.1% TX-100, 5 µg/mL LPS or VC (culture medium) at days 0, 7, 14, 21 and 28. After 24 hr of treatment, the media samples were collected for determination of analytes IL-6 **(A)**, IL-8 **(B)**, MCP-1 **(C)**, TIMP-1 **(D)** and GRO-α **(E)**. The data from VC and LPS treated groups are presented as pg/mL of culture media. N = 6 per group per time point, ****:0.0001<p, ***:0.001<p, **:0.01<p, *0.05<p, two-way ANOVA, Tukey's multiple comparisons test.

hPCLS Histological Evaluation

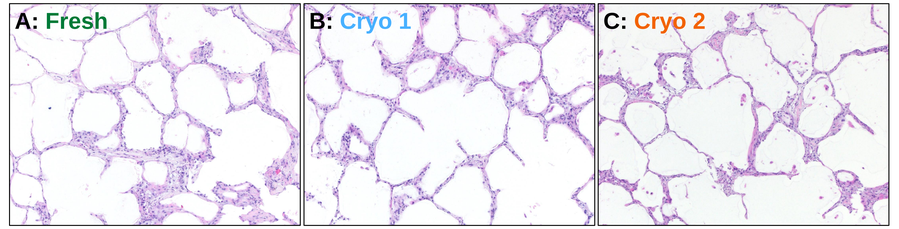

Figure 4. Histological evaluation of cryopreserved hPCLS. The optimized culture methodology applied results in excellent retention of lung architecture and normal cytological features after 29 days culture in fresh hPCLS (**A**). The cryopreservation methods applied retain the tissue integrity and viability without any significant histologic changes in the lung slice tissue after ~7 weeks frozen, thaw and 29 days culture (**B**), and also after ~34 weeks frozen, thaw and 29 days culture (**C**). N = 3 per group.

Histology Scoring

Days	hPCLS type	Treatment	H&E AVE ± SD	ProSurfactant C AVE ± SD	CD86 Intra-alveolar AVE ± SD	CD86 Interstitial AVE ± SD	Aquaporin 5 AVE ± SD
1	Fresh	VC	87 ± 3	14 ± 10	12 ± 4	2 ± 0	200 ± ND
		LPS	80 ± 9	18 ± 12	15 ± 3	3 ± 1	177 ± 25
	Cryo 1	VC	83 ± 8	11 ± 2	29 ± 30	6 ± 2	160 ± 42
		LPS	75 ± 9	8 ± 0	20 ± 9	4 ± 0	177 ± 15
	Cryo 2	VC	60 ± 10	19 ± 3	9 ± 2	4 ± 1	133 ± 25
		LPS	68 ± 8	19 ± 2	15 ± 2	3 ± 1	137 ± 12
8	Fresh	VC	78 ± 3	17 ± 13	16 ± 6	4 ± 1	190 ± 0
		LPS	87 ± 6	6 ± 6	17 ± 10	6 ± 1	203 ± 6
	Cryo 1	VC	85 ± 5	7 ± 2	21 ± 8	5 ± 2	197 ± 6
		LPS	83 ± 3	25 ± 3	12 ± 4	12 ± 1	217 ± 6
	Cryo 2	VC	78 ± 3	16 ± 4	13 ± 2	6 ± 2	193 ± 15
		LPS	75 ± 5	12 ± 4	15 ± 5	8 ± 0	197 ± 23
15	Fresh	VC	82 ± 3	9 ± 6	24 ± 9	7 ± 2	185 ± 5
		LPS	83 ± 6	5 ± 2	13 ± 1	5 ± 0	203 ± 6
	Cryo 1	VC	80 ± 5	12 ± 5	16 ± 5	6 ± 1	197 ± 12
		LPS	80 ± 5	13 ± 6	12 ± 1	7 ± 2	190 ± 26
	Cryo 2	VC	82 ± 3	13 ± 1	22 ± 17	9 ± 4	203 ± 12
		LPS	75 ± 0	10 ± 2	10 ± 2	11 ± 1	207 ± 6
22	Fresh	VC	80 ± 0	5 ± 2	31 ± 23	9 ± 4	177 ± 32
		LPS	80 ± 0	3 ± 1	21 ± 1	8 ± 1	187 ± 25
	Cryo 1	VC	80 ± 0	4 ± 2	18 ± 7	10 ± 1	197 ± 6
		LPS	83 ± 3	6 ± 2	13 ± 2	8 ± 0	213 ± 12
	Cryo 2	VC	80 ± 5	6 ± 5	22 ± 14	11 ± 2	217 ± 6
		LPS	77 ± 6	7 ± 2	15 ± 7	14 ± 1	207 ± 15
29	Fresh	VC	73 ± 3	3 ± 1	14 ± 8	9 ± 1	165 ± 18
		LPS	77 ± 6	3 ± 0	13 ± 6	9 ± 1	178 ± 8
	Cryo 1	VC	82 ± 8	4 ± 2	19 ± 14	9 ± 1	160 ± 20
		LPS	75 ± 5	3 ± 1	31 ± 34	13 ± 5	173 ± 12
	Cryo 2	VC	78 ± 3	3 ± 1	12 ± 3	15 ± 3	203 ± 25
		LPS	78 ± 3	5 ± 3	22 ± 7	12 ± 2	177 ± 15

Table 1. hPCLS histology scores. The hPCLS were stained with H&E (tissue structure) and IHC stains for CD86, Aquaporin 5, and Prosurfactant C. The histology scores for each stain are presented as Average (AVE) ± Standard deviation (SD). N = 3 per group per time point. The histological scoring for H&E stained tissues were performed as follows:
100 – 81%: Tissue appears normal, no histologic evidence of cellular injury other than baseline changes associated with slicing.
80 – 61%: Some pyknotic nuclei, occasional partially denuded alveoli, airway epithelium intact.
60 – 41%: Many pyknotic nuclei, several denuded or partially denuded alveoli, significant loss of overall cellularity, focal loss of airway epithelium.
40 – 21%: Only a few identifiable alveolar lining cells are present. Partially denuded airways.
20 – 0%: Almost complete loss of alveolar lining cell and the airway epithelium.

CONCLUSIONS

- The potential to cryopreserve hPCLS (especially with greatly increased production rates) provides a means to store and maximally utilize the valuable donated human lung tissue and also provides a means to retest the same tissue at a later date.
- Use of appropriate cryopreservation and culture methods can help maintain the cellular and mechanistic functions of the hPCLS for prolonged times.
- Long term hPCLS cultures may allow detection of Key Events involved in Adverse Outcome Pathways of pulmonary disease progression (e.g. fibrosis, respiratory sensitization) that require multiple cell types.
- Effective hPCLS cryopreservation techniques provides a method by which to begin banking tissues of different disease states and/or genetic predispositions so that targeted research can be conducted on population subsets or other select groups.

ACKNOWLEDGMENT

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