Addressing Key Factors in Cryopreservation Method Development Ensures Robust Performance for Human Precision-cut Lung Slices

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ABSTRACT

As new approach methodologies (NAMs) are increasingly explored to identify dependable and accurate non-animal alternatives to predict human toxicities, several 3-dimensional test systems have emerged as excellent models of the human respiratory tract. Among these, human precision-cut lung slices (hPCLS) are considered highly relevant, containing many cell types (including key immune cells), small airways, and respiratory parenchyma in a native architecture configuration. Despite their excellent representation of the deep lung, limited accessibility to fresh (non-transplantable) human donor lungs has hampered the hPCLS model as a mainstream test system for screening efforts and standardization toward regulatory applications. Hence, efforts for cold- and cryo-preservation of hPCLS have emerged as potential solutions that will enable hPCLS access in a flexible manner.

To achieve this goal, a cryopreservation and post-thaw culture method was developed via a progressive testing paradigm that evaluated multiple buffer and media recipes, including some which contained serum. Five different cryopreservation buffer (CB) recipes (several containing human serum) were tested in a comparative manner to determine if any sufficiently protected hPCLS during the freeze and thaw cycles. Testing included evaluation of hPCLS freezing immediately after slicing and also after overnight acclimation using standard cell culture incubator conditions. Post-thaw, two media recipes (E-199- and DMEM/F12-based) were evaluated to ascertain the optimal culture medium to sustain hPCLS cultures. Additionally, two culture methods (air-liquid interface (ALI) and submersion) were tested for up to 28 days, to determine the optimal conditions for hPCLS, post-thaw. Endpoints used to evaluate hPCLS culture health included the WST-8 assay (mitochondrial dehydrogenase activity), total protein content (biomass), and histological evaluation of H&E-stained slice sections (cytomorphology).

Results demonstrated that two CBs and that immediate preservation after slicing produced hPCLS with higher post-thaw viability. Further, while both media recipes and both culture methods maintained slices with high viability for ~2 weeks, the DMEM-F12-based medium and ALI culture method was found superior for cultures lasting up to 3 weeks.

The development of an animal product-free process for the cryopreservation and culture of hPCLS highlights the current state of science in terms of the broad scope of product offerings for in vitro and ex vivo test systems that provide human-relevant results. Applying due diligence to refine the buffer and media recipes used for such systems will provide researchers with more consistent and higher quality products that are ethically sourced.

METHODS

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.

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).

Cryopreservation Method Development Over Multiple Donors:

Donor 1:
- Initial assessment of the candidate CB formulations (CB #1-5).
- Each group n=3 slices was tested for viability on 3 subsequent days (D1-3) post thaw on day 0 (D0) using the WST-8 assay.

Donor 2:
- A second evaluation of the CBs was conducted using n=4 slices.
- Post-thaw culture was increased to D7 and tested for viability on D1-D4 and D7

Donor 3:
- A third evaluation of the CBs was conducted using n=4 slices
- Slices were evaluated using the WST-8 viability assay over D1-D4
- Slices cultured in submerged AM overnight, prior to the freezing process (DS+1) were also evaluated for WST-8 viability over the same D1-D4 period.

Donor 4
- CB formulation #2 was identified for an extended culture of three weeks. With the exception of the day of slicing readout (n=3 hPCLS), 6 slices were sequentially evaluated every 2-3 days for WST-8 viability through D21.
- Additional testing using Donor #4 was conducted approximately 30 weeks after the initial freezing dates.
- hPCLS were cultured under different conditions to compare overnight (ON) vs 3-day (3D), E-199-based medium vs DMEM/F12-based medium, and the method of culture (submerged vs ALI).

CONCLUSIONS

The availability of robust, human-tissue-based non-animal test systems that can be maintained for weeks and potentially reflect key events associated with adverse outcome pathways (AOP) is central to the New Approach Methodology initiative. The effort described here in developing a method by which hPCLS (arguably the most relevant model of the human deep lung) can be cryopreserved, stored for months, and thawed and cultured for 3 weeks or more has demonstrated that animal products (including serum obtained from human donors) are not needed. This stands true for all buffers and media involved, from lung processing and slicing, freezing, and thaw and culture. Subsequent work describes 4 or more weeks post-thaw culture (using the same buffer and DMEM/F12 media recipes) are possible and that the fibrotic phenotype can be maintained after freezing. The establishment of a performance characterization protocol (utilized for every batch of frozen donor slices - normal and diseased) provides a consistent method for understanding the quality of banked hPCLS prior to selection of the appropriate donor(s) for studies.

ACKNOWLEDGMENTS

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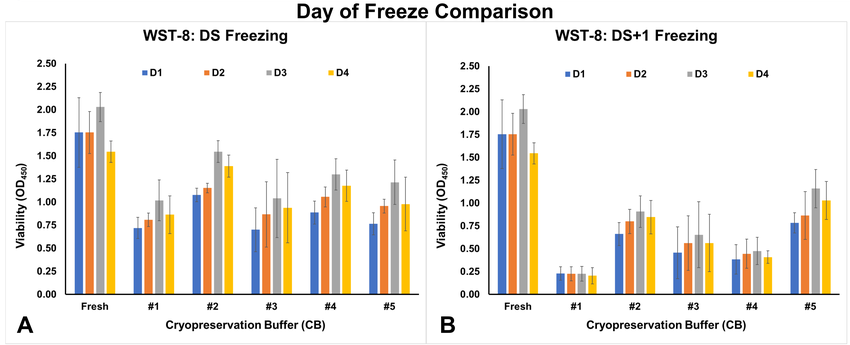
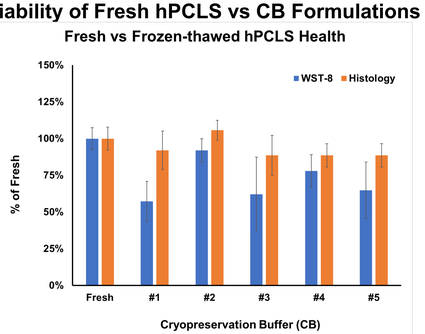
RESULTS

Day of Freeze Comparison



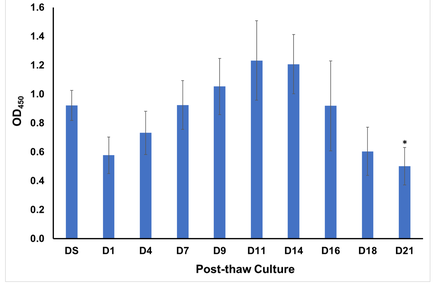
- hPCLS were frozen on the day of slicing (DS) or cultured submerged overnight in E-199 based AM and freezing immediately after (DS+1).
- WST-8 data indicated that freezing on the day of slicing (A), rather than overnight culture and then switch to CB and freezing (B), benefits the tissue viability.

Viability of Fresh hPCLS vs CB Formulations



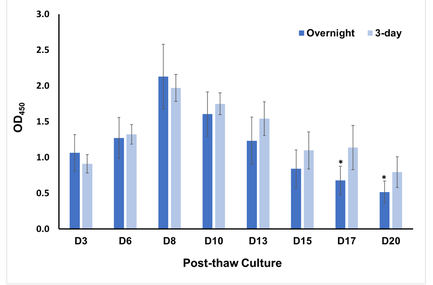
- Fresh WST-8 values and histology scores were set as the reference point (100%) and the performance of the cryopreservation buffer (CB) formulations was compared for both endpoints.
- CB #2 demonstrated the highest viability for both endpoints. N = 3 or 4/group.

Long term viability of CB #2 Frozen-thawed hPCLS from Donor #4



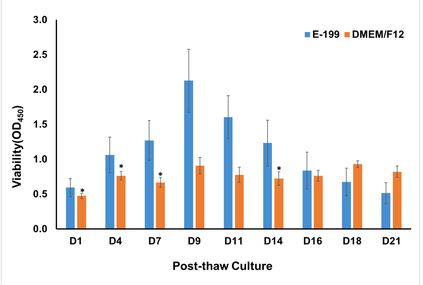
- WST-8 viability was compared with day of slicing (DS) assayed tissues (n=3) and 21-day cultured hPCLS that had been frozen in CB #2.
- Viability values (OD450) improve over time after thaw and culture initiation. Thawed WST-8 values were found to match fresh data (D7) and increase beyond it to 134% of DS by D11. However, after D14 values diminish until D21 values are found significantly lower than the DS group.

Impact of Acclimation Length on hPCLS Viability



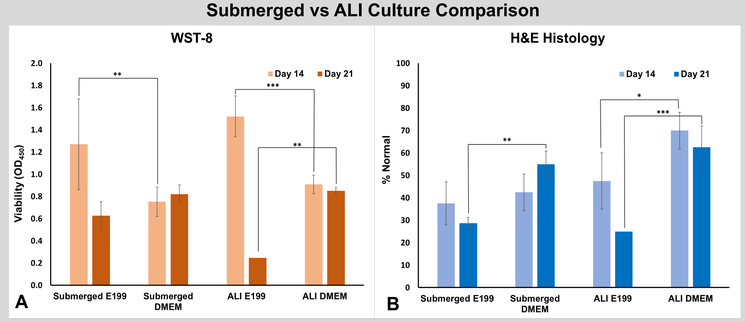
- Overnight (ON) vs 3-day (3D) acclimation periods were compared to ascertain impact on long term cultures.
- Viability reads (WST-8 assay) increased for both ON and 3D groups for approximately 8 days before falling through the remainder of the evaluation period.
- 3D acclimation was found to generate higher performance, especially at later time points, over the 20-day post acclimation sampling period.

Comparison of E-199 vs DMEM/F12 CM in Long Term Cultures



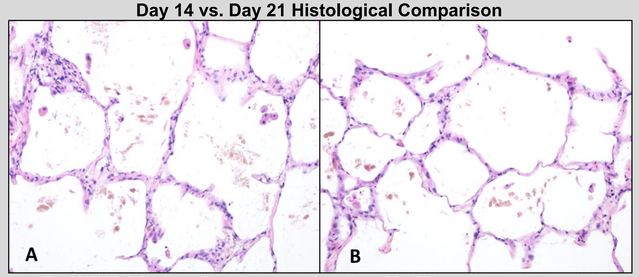
- DMEM/F12-based CM was compared to E-199 CM using frozen hPCLS from Donor #4 , using ON acclimation of respective AM
- DMEM/F12 media based viability reads appeared to plateau and did not continue to decrease as did the E-199 CM group.

Submerged vs ALI Culture Comparison



- Both media groups (E-199 and DMEM/F12 CM) were compared (at days 14 (D14) and 21 (D21)) in submerged vs ALI culture conditions, with evaluation of tissue viability using WST-8 viability (A) and H&E histology (B).
- Submerged WST-8 viability reflects what was previously observed, but the E-199 decline at later time points is more pronounced at ALI.
- Histological evaluation appears to reflect WST-8 viability with the exception that WST-8 viability at D14 appears higher. It was concluded the DMEM/F12 ALI condition was the most conducive for long-term, post- thaw hPCLS viability. Data from different groups were compared using two-way ANOVA with Tukey's multiple comparisons test. N = 2 or 4/group.

Day 14 vs. Day 21 Histological Comparison



- The DMEM/F12 CM ON acclimation group had D14 (A) and D21 (B) photomicrographs taken during histological evaluation.
- The images depict a retention of tissue architecture and cellular viability that are reflective of good lung tissue health. Further, the presence of intra-alveolar macrophages are noted at both days. H&E, 20x objective