IMPACT OF LONG AND SHORT TERM CRYOPRESERVATION ON INTACTNESS OF PRECISION CUT LUNG SLICES

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Introduction

Increasing public awareness of animal welfare and EU legislation have led to a growing interest in alternative models to animal testing [1, 2]. Precision cut lung slices (PCLS) are a model that represent the organotypic 3D architecture of the lung as a very suitable ex vivo cell culture system [3]. They have been successfully used to study various infectious diseases while avoiding animal experiments [4, 5]. In recent years, attempts have been made to investigate the possibilities and limitations of cryopreservation of PCLS in liquid nitrogen. For example, only minor metabolic effects and adverse impacts on contractility of airways were observed in murine PCLS after cryopreservation for up to two weeks [6]. In addition, PCLS from mice and rats that were stored frozen for a few days have been established as a model for chemical toxicological screening using zinc chloride as model toxin [7]. In another study, cryopreserved human PCLS showed only slightly reduced cell viability and a still robust phagocytosis [8]. However, there is a lack of data on more standardized cryopreservation and the effect of the duration of freezing time period on cell viability and function.

Materials and Methods:

Briefly, ferret (n = 9) and porcine (n = 10) lungs were filled with low melting agarose and allowed to solidify on ice. Cylinders of this fixed tissue were cut into approximately 250 µm thick PCLS using the Krumdieck Tissue Slicer (TSE Systems, Chesterfield, MO, USA). The agarose was removed in three washing steps using DMEM/F-12 culture medium in the incubator at 37°C. The next day, PCLS were screened for ciliary activity and subdivided into up to three groups of equal activity. Except for the non-frozen control group (16 PCLS), the two remaining groups consisting each of 16 PCLS were transferred into eight cryotubes. The cryotubes were filled with 1 ml of freezing medium [45% DMEM/F-12, 45% fetal bovine serum, 10% dimethyl sulfoxide (DMSO)] each and slowly cooled to -80°C using Mr Frosty (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). After 3 hours, the cryotubes were stored in liquid nitrogen for 7 or 70 days. After the freezing time period the PCLS were rapidly thawed at 37°C and transferred to culture medium.

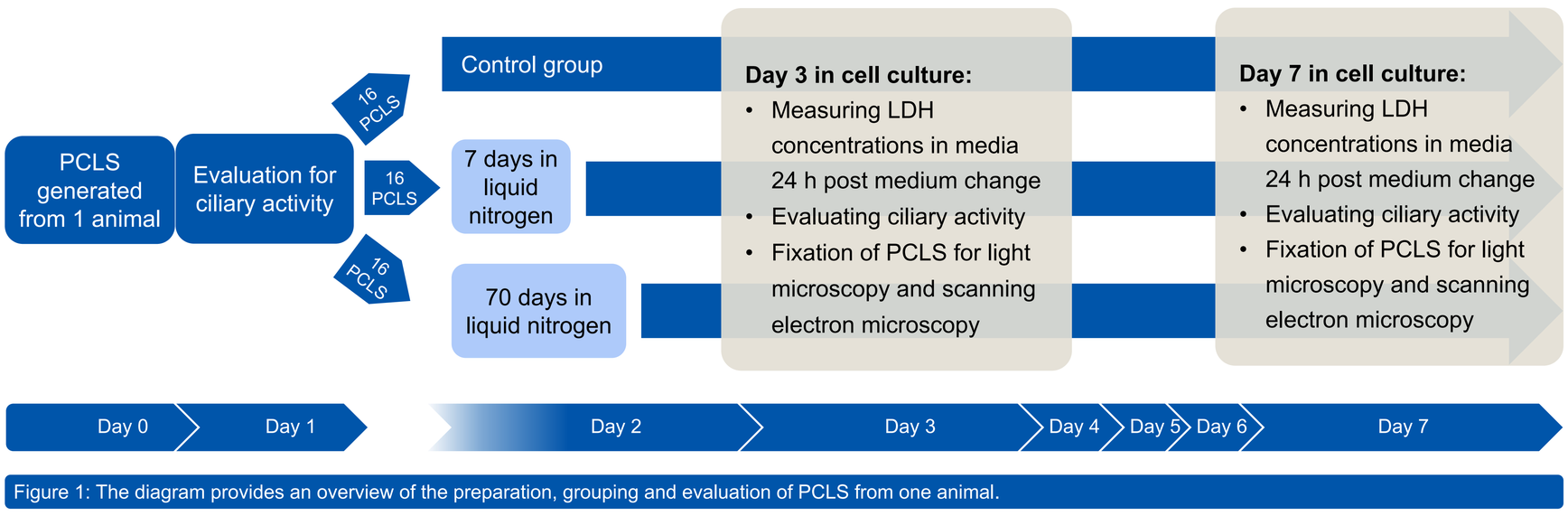

Figure 1: The diagram provides an overview of the preparation, grouping and evaluation of PCLS from one animal.

On the third and seventh day in culture, the PCLS were evaluated by determining ciliary activity and measuring lactate dehydrogenase (LDH) concentration in the supernatant, which had been replaced 24 hours earlier. As a positive control, two PCLS were incubated in 200 µL PBS with 1% Triton at 4°C for 1 h. At least four additional PCLS were fixed for histology and scanning electron microscopy (SEM) at the above-mentioned time points. 15 serial sections were prepared from each formalin fixed, paraffin-embedded PCLS. Five representative sections were stained with H&E (hematoxylin and eosin) and the percentage of cells with cilia, regressive nuclear changes and cytoplasmic vacuolization compared to the total number of cells in the bronchial epithelium was determined. Cilia in the bronchus were labelled in two serial sections for α-tubulin using immunohistochemistry and the number of positive cells was counted in two high-power fields (400x). Following the osmium (O)-thiocarbohydrazide (T) embedding protocol (OTOTO), PCLS were sputter-coated with 15 nm gold for SEM. Thereafter, the number of cells with cilia was counted in relation to the total number of cells in eight images (4000 x) per time point.

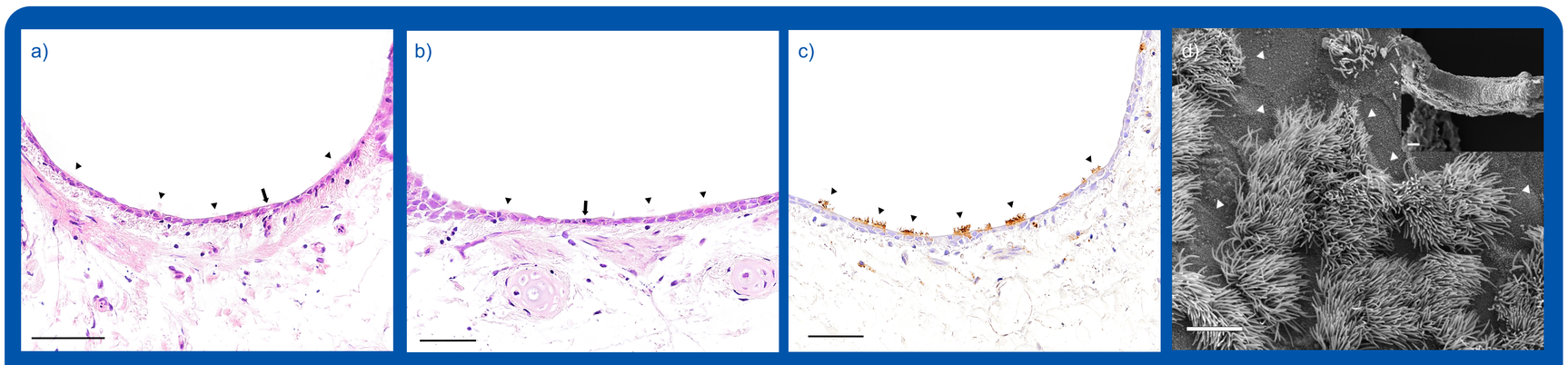

Figure 2: Cryopreserved (a, d: 7days frozen; b, c: 70 days frozen) Precision cut lung slices (PCLS) show cilia (arrowheads) on the apical side of respiratory epithelium in H&E staining (a, b), as well as in immunohistochemical staining for α-tubulin (c). In single cells vacuoles (a, arrow) or regressive nuclear changes (b, arrow) were visible. Scanning electron microscopy provides a more detailed view of cilia (arrowheads) from the luminal side (d). All shown PCLS are ferret derived and under culture conditions 3 days. Scale bar: a, b, c: 100 µm; d: 10 µm; d insert: 100 µm

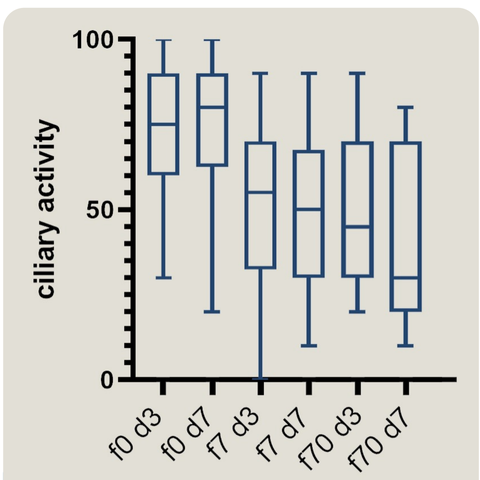

Figure 3: The ciliary activity of PCLS of a representative ferret. (f = days in liquid nitrogen; d = days under culture conditions)

Results

In the comparative analysis of ferret and porcine PCLS, ciliary activity was observed in both cryopreserved and non-cryopreserved groups. However, a statistically significant adverse effect on PCLS was observed for the freezing period of 7 and 70 days as well as the culture conditions from day 3 to day 7. But there was no significant difference between the 7-day and 70-day freezing periods. The same significant effects of cryopreservation and culture conditions were seen with LDH concentration, another vitality parameter. It increase slightly between the third and seventh day under culture conditions in frozen and non-frozen PCLS. In addition, there was a significant decrease in LDH concentration in the cryopreserved PCLS compared with the control group, which could be due to a reduced number of viable cells after storage in liquid nitrogen. But there was no significant effect of the duration of cryopreservation on LDH concentrations. Pathomorphological evaluation of H&E-stained ferret PCLS showed cilia in the respiratory epithelium in the frozen and control groups, as well as single cells in the epithelium with regressive nuclear changes or vacuoles in the cytoplasm. Ciliated cells were also detected by immunohisto-chemistry staining for alpha-tubulin and by scanning electron microscopy.

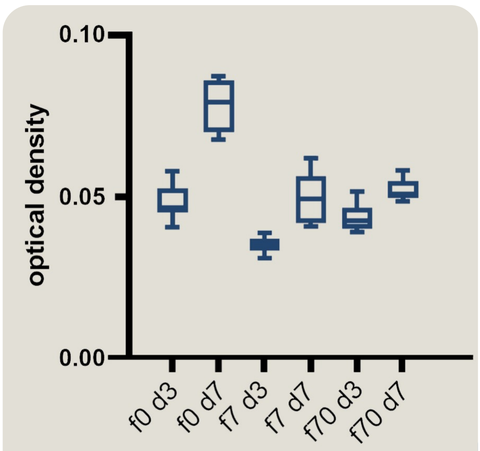

Figure 4: The optical density after the LDH detection reaction is proportional to the concentration in the culture medium of PCLS from a representative ferret. (f = days in liquid nitrogen; d = days under culture conditions)

Conclusion

Cryopreservation and culture conditions appear to have significant effects on the ciliary activity and viability of the PCLS. Given these limitations cryopreservation allows for more flexible use of the PCLS as a tool to study infectious diseases and toxic effects.

References

1. Anonymus. Meinungen zu Tierversuchen in der Medikamentenforschung. 2017 19.02.2023]; Available from: https://www.aerzte-gegen-tierversuche.de/de/tierversuche/statistiken/2362-umfrage-2017-mehrheit-gegen-tierversuche.
2.Anonymus, Richtlinie 2010/63/EU des Europäischen Parlaments und des Rates vom 22. September 2010 zum Schutz der für wissenschaftliche Zwecke verwendeten Tiere. 2010.
3.Viana, F., C.M. O'Kane, and G.N. Schroeder, Precision-cut lung slices: A powerful ex vivo model to investigate respiratory infectious diseases. Molecular Microbiology, 2022. 117(3): p. 578-588.
4.Runft, S., et al., Alternatives to animal models and their application in the discovery of species susceptibility to SARS-CoV-2 and other respiratory infectious pathogens: A review. Veterinary Pathology, 2022. 59(4): p. 565-577.
5.Vötsch, D., et al., Bordetella bronchiseptica promotes adherence, colonization, and cytotoxicity of Streptococcus suis in a porcine precision-cut lung slice model. Virulence, 2021. 12(1): p. 84-95.
6.Rosner, S.R., et al., Airway Contractility in the Precision-Cut Lung Slice after Cryopreservation. American Journal of Respiratory Cell and Molecular Biology, 2014. 50(5): p. 876-881.
7.Watson, C.Y., et al., Screening for Chemical Toxicity Using Cryopreserved Precision Cut Lung Slices. Toxicological Sciences, 2016. 150(1): p. 225-233.
8.Bai, Y., et al., Cryopreserved human precision-cut lung slices as a bioassay for live tissue banking. A viability study of bronchodilation with bitter-taste receptor agonists. American Journal of Respiratory Cell and Molecular Biology, 2016. 54(5): p. 656-663.

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