Toxic Insult to Rat Precision Cut Lung Slices Increases Tissue Cytokine Levels and Activation of Macrophages, and Causes Acute Damage, While Prolonged Insult May Lead to Increased Deposition of Collagen - a Marker of Fibrosis

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ABSTRACT

The use of in vitro or ex vivo models is intended to provide meaningful data that will identify or predict the adverse effects of tissue exposure. Precision-cut lung slices (PCLS) are used as a model that retains the heterogenous population of cells in the native architecture of the organ. The retention of native cells allows the study of the initial, dynamic events (such as inflammation) that occur following a toxic insult prior to overt tissue damage. The purpose of the reported studies was to identify initial inflammatory signals, acute toxicities, as well as markers associated with chronic toxicities of PCLS exposed to a toxic insult as a way to qualify the model for identifying such endpoints. Rat PCLS were exposed to several chemotherapeutics known to cause acute and/or chronic pulmonary damage. Time points for respective endpoints were chosen based on known response times of when relevant endpoints may change. Cytokines and acute toxicity were evaluated during initial days of exposure while activation of macrophages and collagen deposition were evaluated through 4 weeks of culture in other studies. Exposure of PCLS for 24 hours resulted in increased cytokine levels and 72 hour exposure caused overt toxicity, as assessed using tissue protein content and histologically using H&E and ED-1 staining. Long term exposure of PCLS to two agents known to cause fibrosis (bleomycin and carmustine) resulted in elevated numbers of macrophages and also increased collagen deposition. PCLS generate inflammatory cytokine signals and, if levels persist after insult removal, these signals may predict subsequent tissue damage. The expression of adverse markers of chronic exposures (collagen deposition) in PCLS may signify risk of fibrosis. Cytokine responses, macrophage activation, and fibrosis are hallmarks of tobacco related exposures. PCLS may elucidate acute and chronic adverse pulmonary responses when exposed to tobacco products.

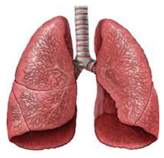
INTRODUCTION

With the expanded regulation of tobacco products and the need to assess inhaled toxicants, researchers require models that allow accurate translation of results. *In vivo* models are not always suitable for mechanistic studies and with the 3Rs initiative to expand the use of *in vitro* models, researchers are often attracted to more complex 3-dimensional (3D) models that offer a heterogeneity of cell types for more diverse cell-cell signaling.

Exposure of lungs to tobacco smoke, or modified risk tobacco products, results in a series of events that can include inflammation leading to acute damage, or with repeated exposure, may result in a chronic inflammatory state that may ultimately lead to fibrosis and/or chronic obstructive pulmonary disease. The complex sequence of events involves many cell types and diverse signaling between parenchyma and mediators of inflammation. While several 3D *in vitro/ex vivo* airway epithelium models can offer multiple cell types, only *ex-vivo* precision-cut lung slices (PCLS) are known to retain macrophages – a cell known to have a central role in pulmonary inflammation.

This study reviews PCLS as a model that demonstrates longevity in culture, responds to challenge by regulating cytokines, demonstrates acute damage, and also expresses biomarkers associated with chronic toxicity.

MATERIALS & METHODS



1. Inflate lung tissue and create tissue cores

- Aseptic lung removal and storage in organ preservation solution. Inflation with 0.8% agarose, lobe dissociation, and tissue coring (8 mm).

2. Slice cores with Krumdieck slicer

- In thermostatically controlled cold UW, cores are sliced to 500 micron thickness

3. PCLS placed in vials

- Slices are mounted onto HATF paper in titanium inserts, placed in vials and cultured in 1.7 mL serum-free, M199 medium

4. PCLS are acclimated

Vials are rotated at ~3-7 rpm in roller drum within humidified incubator set to 5% CO2/95% air at 37ºC

5. Culture maintenance

After acclimation period, inserts are transferred to vials containing treatment medium (replaced every 1-2 days); Medium is collected through a slice's lifespan until harvest.

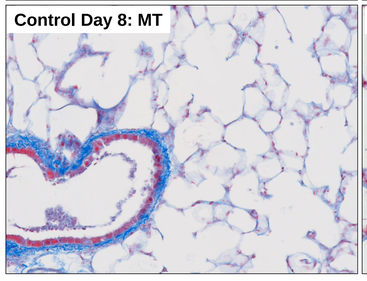
6. PCLS harvest:

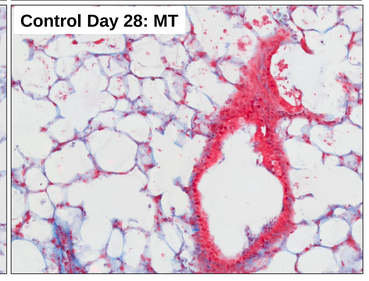
a) PCLS for biochemical evaluation are homogenized in 500 µL ice cold PBS+ 0.5% Triton X-100

b) PCLS for histological evaluation are fixed with 4% paraformaldehyde for ~24 hr, transferred to 70% EtOH, embedded, sectioned and stained

RESULTS

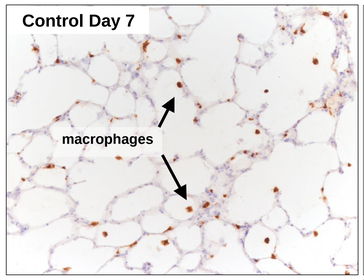
PCLS Culture Longevity

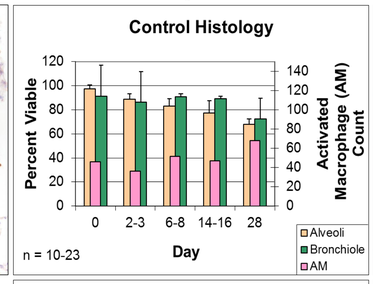




- Alveoli are patent and bronchiolar epithelium is intact. Only few strands of collagen fibers in alveolar walls; bronchiole shows thick layer of collagenous tissue
- Histology is well-preserved and bronchiole retains intact epithelial lining cells; normal pattern of collagen deposition within interstitium *(MT = Masson's Trichrome)*

Viability and Biochemical Stability



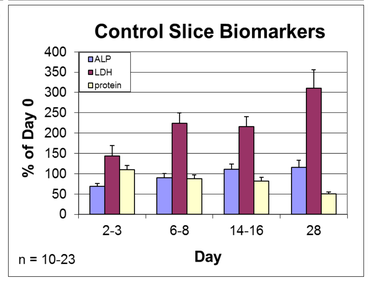


Viability and macrophages

- Control slices exhibit baseline numbers of activated macrophages (AM) *(ED-1 immunostaining)*
- High degree of alveolar and bronchiolar viability retained over 28D

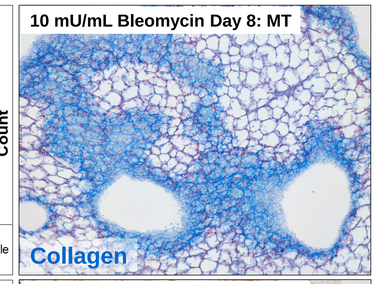
Biomarkers

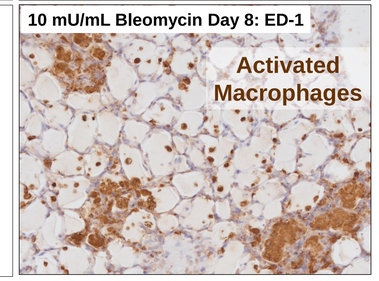
- Retention of tissue markers over 28 days in serum-free M-199 medium
- Some loss of protein over time (coincides with minor loss of cellularity)



- ALP, a marker of Type II alveolar cells remains stable (normalized to tissue protein content) over the entire 28 day culture period

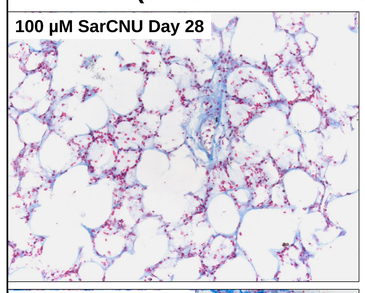
Biomarker Induction

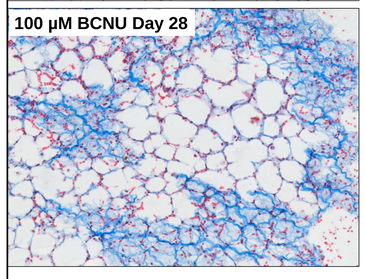




- Bleomycin treatment results in collagen deposition and patches of activated macrophages filling alveolar spaces; many solitary macrophages are also seen

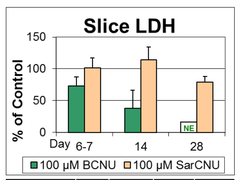
Differential Molecular Analog (SarCNU and BCNU) Toxicity





- Extensive blue, wavy collagenous fibers with intervening normal alveoli

- Comparison of two analogs accurately demonstrates differences in toxicity
- PCLS biomarker content, numbers of AM, and collagen deposition all reflect greater BCNU toxicity vs SarCNU

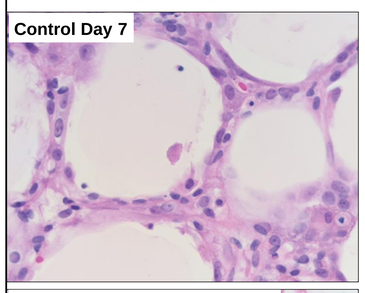


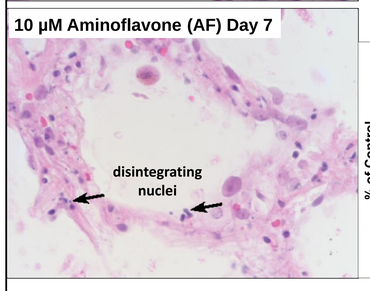
Activated Macrophages in Lung Slices

Compound	µM	Counts [a] Day 6-7	Day 14	Day 28
Vehicle	0	52	48	59
BCNU	1	60	94**	-
	10	67*	71*	-
	100	66	102**	81*
SarCNU	1	56	52	-
	10	61	57	-
	100	69*	76*	71

[a] Means of 3 measures on 3-4 replicates
* p<0.05 ** p<0.01

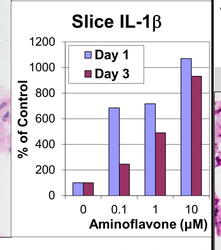
Cytokine Induction Precedes Cell Death in Human PCLS





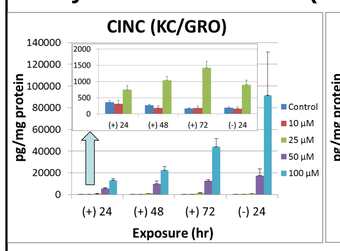
- AF-induced, decreased cellularity and nuclear changes reflecting toxicity

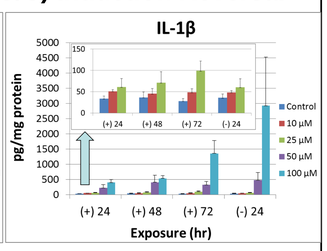
- Control tissue shows alveoli lined by mostly viable cells
- Exposure of human PCLS to 10 µM AF causes cytokine increases in < 24 hr
- Days later, severe tissue damage was noted



- Concentration-depend. IL-1β increases

Identification of No Effect Level and Cytokine/chemokine (C/C) Induction Reversal

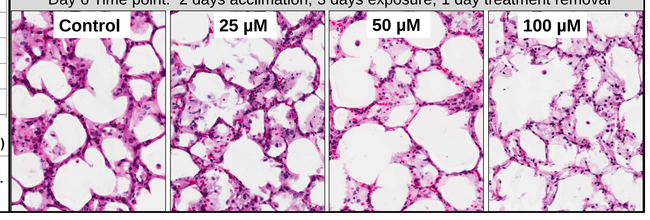




- Phortress at 10 µM (not shown) has no effect while 25 µM shows minimal evidence of toxicity histologically.
- With 50 and 100 µM Phortress, PCLS show decreased cellularity; cells lining the alveoli display pyknotic nuclei

- Initial C/C level increases of PCLS treated with 25 µM subside after Phortress removal
- Despite removal of drug, C/C levels continue to increase in PCLS treated with 50 and 100 µM Phortress

Day 6 Time point: 2 days acclimation, 3 days exposure, 1 day treatment removal



CONCLUSIONS

1. PCLS exhibit longevity and retention of viability for 1 month or more. This makes them suitable for long term culture and repeated exposure paradigms in a manner that can reflect consumer product use (e.g. tobacco product exposures over time).
2. The 3D, native lung parenchymal architecture, and inclusion of native cell types allows for a complex response (e.g. activation of macrophages, increased cytokine expression, and collagen deposition) to challenge. The involvement of multiple cell types and biomarkers may be required for a long term disease such as COPD.
3. PCLS have historically been employed to compare compound toxicities and the model has repeatedly demonstrated differential effects and severity of response across the compounds tested. By extension, PCLS are well suited to make specific tobacco product or product combination comparisons.
4. The identification of a no adverse effect level and a level at which increased inflammatory markers subside after treatment removal will help guide product dosimetry decisions.

REFERENCES

- Behrsing, H. P., et al. (2013). In vitro exposure of precision-cut lung slices to 2-(4-amino-3-methylphenyl)-5-fluorobenzothiazole lysylamide dihydrochloride (NSC 710305, Phortress) increases inflammatory cytokine content and tissue damage. Toxicol Sci 131, 470-9.
- de Kanter, R., et al. (2002). Precision-cut organ slices as a tool to study toxicity and metabolism of xenobiotics with special reference to non-hepatic tissues. Curr Drug Metab 3, 39-59.
- Hay, J., et al. (1991). Mechanisms of bleomycin-induced lung damage. Arch Toxicol 65, 81-94.
- Placke, M. E., and Fisher, G. L. (1987). Adult peripheral lung organ culture--a model for respiratory tract toxicology. Toxicol Appl Pharmacol 90, 284-98.
- Schuller, H. M., et al.. (1985). Sequential pathological changes induced in rats with the anti-cancer drug I, 3-bis (2-chloroethyl)-I-nitrosourea (BCNU). Exp Lung Res 9, 327-39.
- Pfaller, W., et al. (2001). Novel advanced in vitro methods for long-term toxicity testing: the report and recommendations of ECVAM workshop 45. European Centre for the Validation of Alternative Methods. Altern Lab Anim 29, 393-426.

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