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The Spatial Response of Penh as a Secondary Readout for the Airway Hyper-reactivity in Three Different Rodent Models of Airway Inflammation: A Pilot Comparison Study

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Authors' contributions

This work was carried out in collaboration between all authors. Authors DT and PCC designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript and managed literature searches. Author JDW managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Inflammatory cell infiltration, increase airway hyper-reactivity, and inflammatory cytokine production are all hallmarks of pulmonary diseases such as asthma and chronic obstructive pulmonary disease (COPD). While cytokine production and cellular infiltrations are relatively easy to assess in preclinical modeling for drug PK/PD, airway hyper-reactivity is more difficult to model and measure. Enhanced pause (Penh) is a non-dimensional parameter associated with the characteristic changes in the respiratory cycle. It had been used widely in the pulmonary field as a surrogate marker for airway hyper-reactivity (AHR). However, this parameter is not always suitable for measurement of the AHR response. Furthermore, the model sensitivity to Penh is not well understood and need to be further evaluated and documented. In our study, we evaluated the

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suitability of using Penh as measurement in three common rodent airway inflammation models as a potential secondary readout without potentially influencing the primary readout.

Keywords: Inflammation; PK/PD; Airway hyper-reactivity; Penh; secondary; marker; rodent.

1. INTRODUCTION

COPD and asthma are complex, multifactorial airway diseases that affect millions of people worldwide. They are characterized by a strong lung inflammatory component with inflammatory cell infiltration, cytokine production, and airway hyper-reactivity (HSA). Pulmonary disease populations are continuously increasing worldwide. According to the most recent data published by CDC in 2015, in the United State, more than 8% of the Children and 7% of the adult suffer from asthma. For these patients, glucocorticoids are often prescribed as first-line therapy to control symptoms, improve lung function, and reduce morbidity and mortality [1]. Despite the treatment, symptoms for a some patients are not well controlled; furthermore, the ICS side effects are widely reported [1,2,3,4,5]. Due to the above reasons, the pharmaceutical industry is constantly seeking better and safer treatments for the pulmonary diseases. A major challenge to the identification of pulmonary drug candidates is to demonstrate preclinical efficacy in appropriate animal models with higher throughputs. Mouse models are commonly used to study mechanisms of allergic airway inflammation due, in part, to the vast resources of induced mutant strains that can implicate specific gene products in pathogenesis [6-9]. Lipopolysaccharide (LPS), ovalbumin (OVA), and cigarette smoke are widely used to induce airway inflammation in order to model certain aspects of human disease in vivo and determine efficacy of drug candidates [10-14]. While no single model accurately depicts human disease, each model can be used to measure clinically relevant markers or endpoints. For example, common techniques for evaluating such determinants include the collection of bronchoalveolar lavage (BAL) fluid and blood. The BAL fluid is often used for quantifying cellular infiltrates and levels of cytokines and chemokine. Likewise, the blood can also be used for *ex vivo* analysis of cytokines / chemokines including the determination of TNF-α levels [15,16]. The major drawback of these end points, particularly the collection of BAL fluid, is that they are terminal and do not provide a spatial representation of the disease progression or treatment effects. Furthermore, the analysis of these samples is often time consuming and does not support the real time decision-making process. On a mechanistic level, these biomarkers can only serve as surrogates for the effects of treatment on lung function. Also, due to the terminal nature of these read outs, the amount of data that can be obtained from one study is limited. Thus, non-invasive and non-terminal end points are extremely valuable as potential secondary readouts.

Enhanced pause (Penh) is a non-dimensional parameter defined by the ratio of the exhalation pulmonary pressure to the inhalation pulmonary pressure, and the ratio of the exhalation cycle duration to 36% of the exhalation cycle. Penh reflects changes in the wave form of the air pressure signal from both inspiration and expiration, and combines it with the timing comparison of early and late expiration (pause). Penh = (*Te*/*Tr* -1) (PEF/PIF), where *Te* is expiratory time (the time from the end of inspiration to the start of the next inspiration), *Tr* is relaxation time [the time of pressure decay to 36% of the total expiratory pressure signal (area under the box pressure signal in expiration)], PEF is peak expiratory flow (ml/s), and PIF is peak inspiratory flow (ml/s). Increases in Penh are related to an increase in pulmonary resistance [6,9]. It is exaggerated during challenge with increasing doses of methacholine (Mch), usually in incremental increase up to 50 mg/ml [6]. This parameter is measured by a full body plythesmograph. It is reported that Penh correlates with pulmonary airflow resistance or obstruction [16]. Penh as measured by plethysmography has been validated in animal models of airway hyper-responsiveness [16-21]. Penh had been used widely in the pulmonary field as a surrogate marker for airway hyper-reactivity (AHR). However, this parameter is not always suitable for measurement of the AHR response. Even though some investigators had questioned its validity, its non-invasive nature enable it serves as an additional readout on top of the primary readouts. It is non-invasive and can be monitored easily during the in vivo experiment without affecting the primary readout. Furthermore, it provides continue data inputs throughout the whole duration and potentially can be used for other exercises that require more data inputs such as PK/PD investigation. We believed that Penh is still a potential secondary readout in the drug discovery setting for

compound selection for pulmonary diseases. We tested the suitability of utilizing Penh as a surrogate marker for the measurement of airway hyperreactivity severity on three different mouse lung inflammation models with a robust time course study.

2. METHODS

2.1 Reagents and Materials

96-well polypropylene plates were purchased from Corning Inc. (Corning, NY). Peri Proneb ultra compression nebulizer was purchased from Peri Co. (Midlothian, VA) and Hamilton dosing needle (IN) and syringe were purchased from Hamilton Co. (Reno, NV). The water purification system used was a Millipore milli-Q system. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were used without further purification.

2.2 Penh Measurement

The Penh measurement was conducted with a Buxco full body plythesmograph. (Need Buxco CO location) Briefly, conscious animals were individually housed in polycarbonate plythesmographic chambers. The chamber pressure was continuously monitored during the observation period. An increasing concentration of Mch was nebulized over the course of the experiment to amplify the Penh signal, and decrease the signal to noise ratio. The respiratory pattern of the animals was recorded, and the Penh response was calculated by the Buxco (DSI, MN, USA) plythesmograph algorithm from the pressure parameters collected by the system.

2.3 Animals

The Institutional Animal Care and Use Committee (IACUC) reviewed and approved the animal use in these studies. The Association for Assessment and Accreditation of Laboratory Animal Care, International fully accredits the Pfizer animal care and use program. Male and female Balb/c and female C57Bl/6 mice were obtained from Jackson Labs and house internally for 2 weeks prior the experiment.

2.4 Lipopolysaccharides Induced Lung Inflammation Model

LPS (*E. coli* O111: B4) was purchased from Sigma-Aldrich (St Louis, MO) and prepared in phosphate buffered saline solution (PBS). Male Balb/c mice at 6-8 weeks old were challenged with 50 μg of LPS (Sigma) in 50 μl by intranasal (IN) administration. The LPS solution was presented to the naris of the animals drop wise. At 4 hours post LPS challenge, the animals were put into the Buxco full body plythesmograph to measure their Penh response. A second Penh measurement was performed 28 hours post LPS challenge (Fig. 1).

2.5 OVA Induced Airway Hypersensitivity Model (OVA/OVA and OVA/Saline)

The OVA induced AHR and airway inflammation model is characterized by Th2 cytokine production, inflammatory cell infiltration (especially eosinophils), and airway hyperreactivity. 6-8 weeks old male Balb/c mice were immunized on day 0 with 40 ug OVA + 4 mg $AlOH_3$ /0.5 ml saline by IP injection (referred as OVA/saline challenged). A booster inoculation was given IP on day 6 along with an IN challenge of 2 mg/mL OVA. 24 hours before the Penh measurement, the animals were challenged for a second time intranasally with 2 mg/mL OVA. Measurements of Penh were obtained on day 24, 25, 26, and day 27 (Fig. 2).

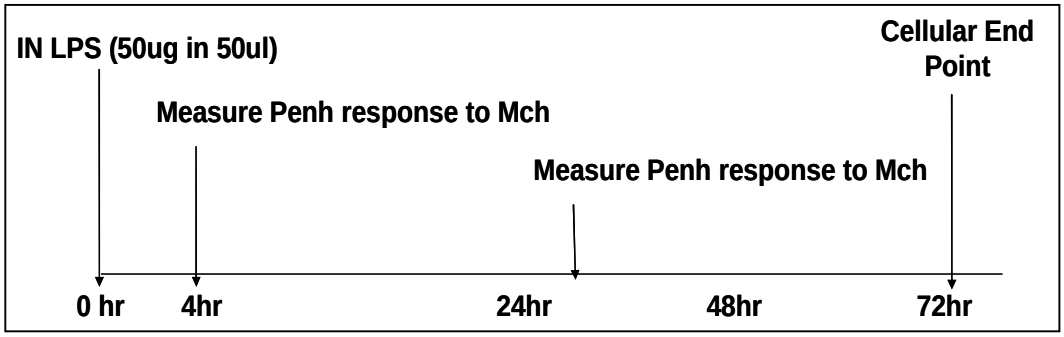

Fig. 1. Timeline of LPS exposures and Penh measurements in mouse LPS model

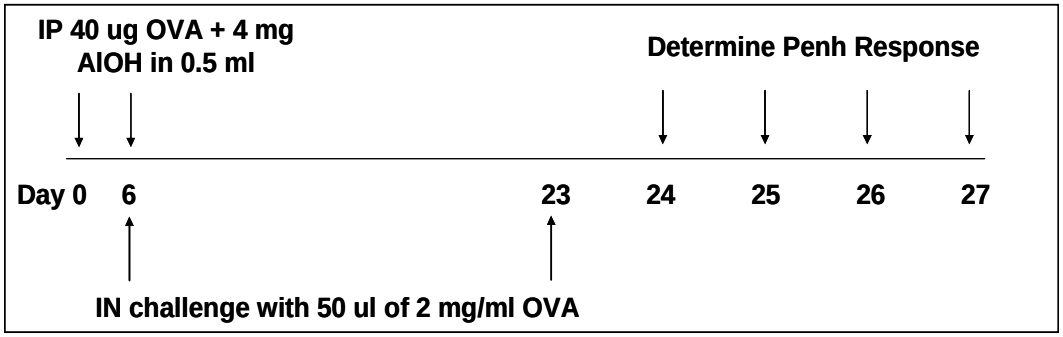

Fig. 2. Timeline of OVA exposures and Penh measurements in mouse OVA model

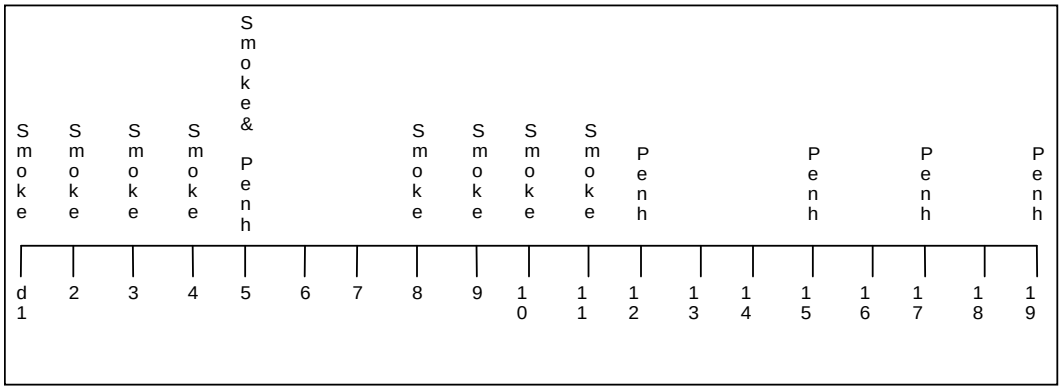

Fig. 3. Timeline of smoke exposures and Penh measurements in mouse cigarette smoke model

2.6 Cigarette Smoke Induced Lung Inflammation Model

Female C57Bl/6 and Balb/c mice 8-10 weeks old, were double-housed within stainless steel, metal mesh cage units and placed in a vertical flow plexiglas chamber. The mice (n=8 per group) were exposed whole body to mainstream and sidestream cigarette smoke generated from 2R4F cigarettes (University of Kentucky) that were loaded onto a Jaeger-Baumgartner 2080 cigarette smoke generator (CH Technologies, USA). Smoke exposure was administered for 2 hours per day on days 1-5 and 8-11 of the study at a target concentration of 400 mg/m^3. Exposure concentration was quantified via gravimetric analysis. Control mice (n=15 per group) received fresh air only as confirmed by gravimetric analysis. The animals were put into the full body plythesmograph to measure a Penh response on day 5, 12, 15, 17 and 19 (see above Fig. 3).

3. RESULTS

The three models of lung inflammation were successfully executed according to the protocol timeline described above. The Penh response was elicited by increasing doses of Mch aerosol challenge. AHR was assessed using a Bucxo Full Body Plythesmograph.

3.1 LPS Model

For the LPS induced airway inflammation model evaluation, Penh was found to be a very sensitive measurement. A significant difference was found comparing Naïve vs. LPS challenge mice. At 4 hours post dose, the baseline Penh was 0.06 for Naïve compared with 0.4 for the LPS challenged mice. The difference between the challenged and naïve animals become more prominent when the animals were challenged with escalating doses of Mch for both the Naïve and sensitize mice (T test). The maximum Penh value was 6.7 for the sensitized mice whereas that for the Naïve mice was 3.6. The peak Penh response was achieved for sensitized mice when challenged with a Mch aerosol generated from a 25 mg/mL solution and remained unchanged with higher concentration. For the Naïve, the peak Penh response began to plateau when 25 mg/ mL solution was nebulized and the maximum response was achieved when challenged with a

Mch aerosol generated from a 50 mg/mL Mch solution (dose response curve is illustrated as Fig. 4). A spatial measurement of the response showed that the window between sensitized and naïve animals was lost at 28 hours post challenge (i.e. at the same time the following day). This was not a surprised finding since the spatial up regulation of other markers such as serum TNF level is very consistent with the above readout (22) (dose response curve is illustrated as Fig. 5).

3.2 OVA Model

For the OVA induce airway hypersensitivity model evaluation, Penh was found to be a responsive parameter. The Penh values of the OVA sensitized and challenged group started to increase significantly 24 hours post challenge over the naïve animals when stimulated with Mch. The window between OVA sensitized and challenged animals and the naïve animals was the greatest at 72 hours post challenge. A significant difference ($P<0.05$) was found comparing Naïve vs. OVA/OVA challenge mice but not the single challenged (OVA/saline) mice. The differences become more significant when the animals were challenged with escalating Mch concentrations for both Naïve and sensitize mice. At 24 hours, the maximum Penh value of 7.1 was obtained for the OVA/OVA sensitized mice whereas the Naïve mice was only 3.7. The peak Penh response was achieved for OVA/OVA sensitized mice was obtained from a Mch aerosol generated from a 25 mg/mL solution and remained unchanged for higher Mch concentration. For the Naïve mice, the peak Penh response began to plateau when the 25 mg/mL solution was used and maximum response was reached with a Mch aerosol generated from a 50 mg/mL solution ($P<0.05$). The OVA/saline sensitized mice followed a similar pattern with Penh reaching 4.9 when challenged with a 50 mg/mL Mch solution. However, the separation from the naïve response was not as significant (dose response curve is illustrated as Fig. 6). The duration of the response was also studied. The experiment was repeated at 48, 72, and 96 hours. It was found the Penh response was very consistent over this time course. At 96 hours, the magnitude of the response remain a roughly the same as 24 hours post OVA aerosol challenge. At 96 hours, the peak Penh response of 7.8 was achieve for OVA/OVA sensitized mice when challenged with a Mch aerosol generated from a 50 mg/mL solution. For the Naïve mice, a peak Penh response of 3.2 was obtained from a Mch aerosol generated from a 20 mg/mL solution. The OVA/saline sensitized mice followed a similar pattern and Penh reaching 4.3 when challenged with a 25 mg/mL Mch solution, again the separation from naïve was not as prominent. An example of the 96 hours Penh response (dose response) is illustrated in Fig. 7.

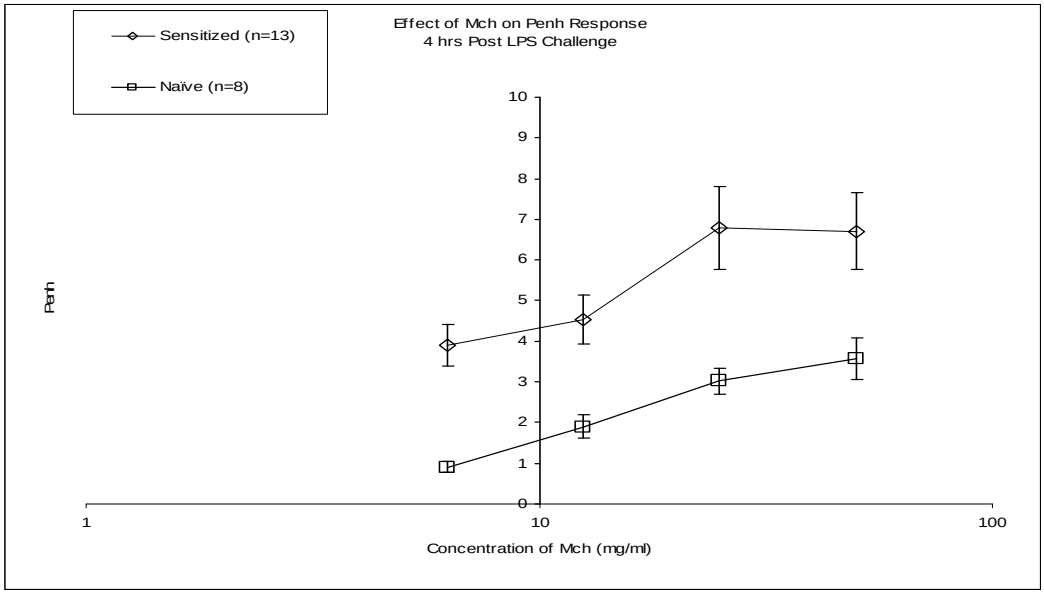

Fig. 4. Effect of Mch on Penh response on LPS model (4 hour post dose)

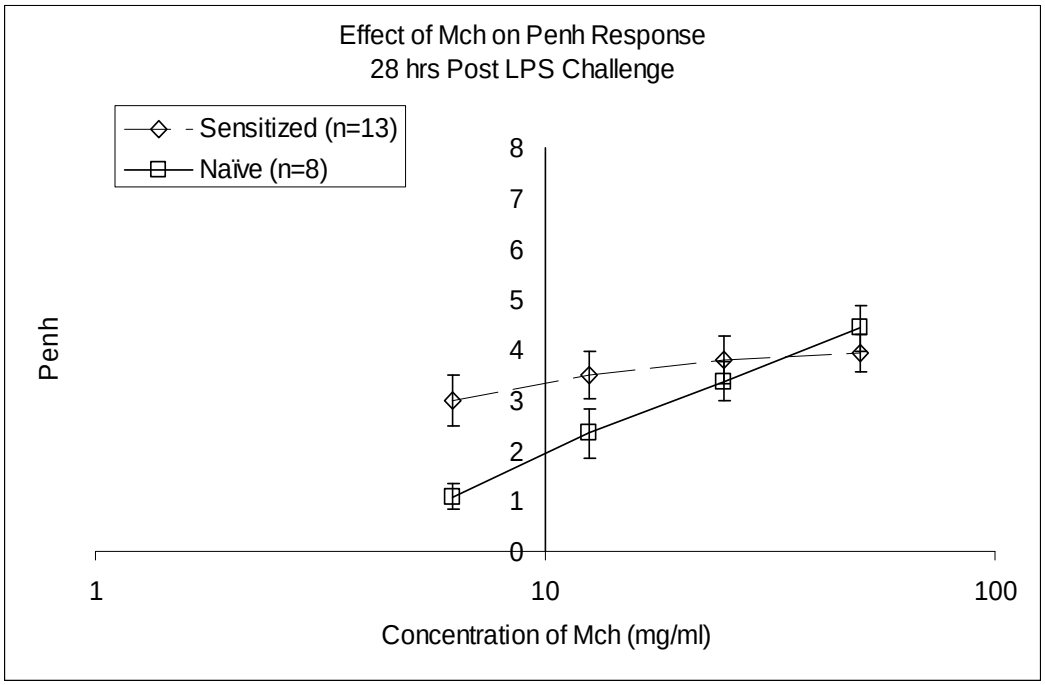

Fig. 5. Effect of Mch on Penh response on LPS model (28 hrs post dose)

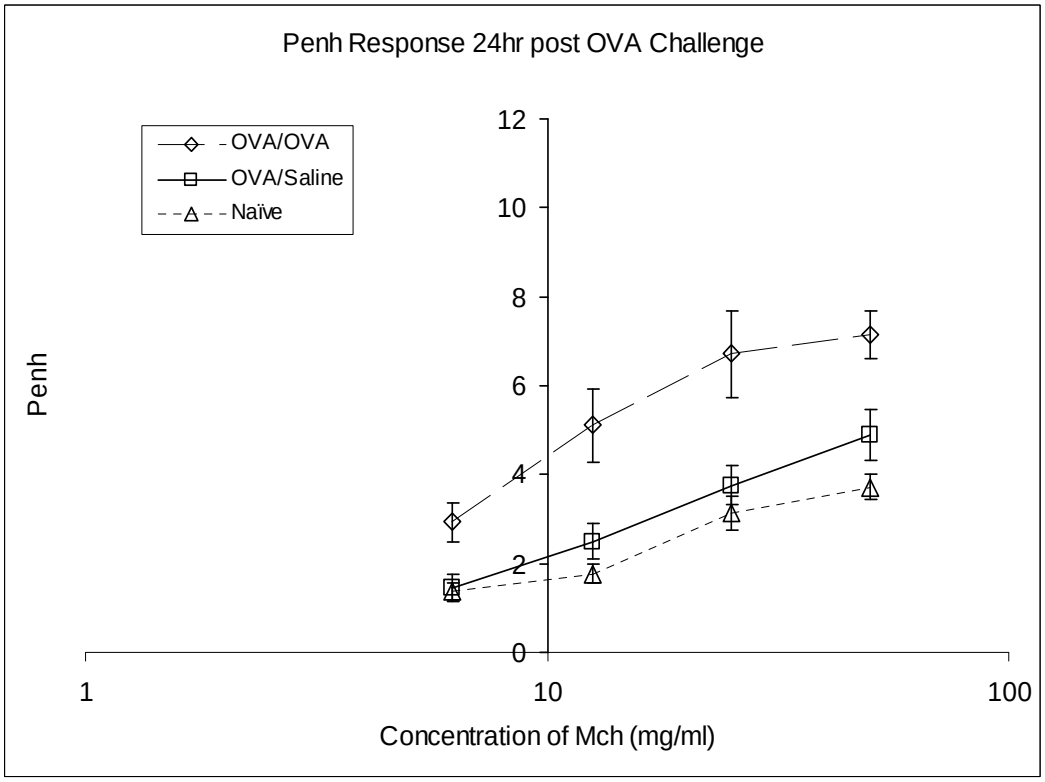

Fig. 6. Effect of Mch on Penh response on OVA model (24 hrs post dose)

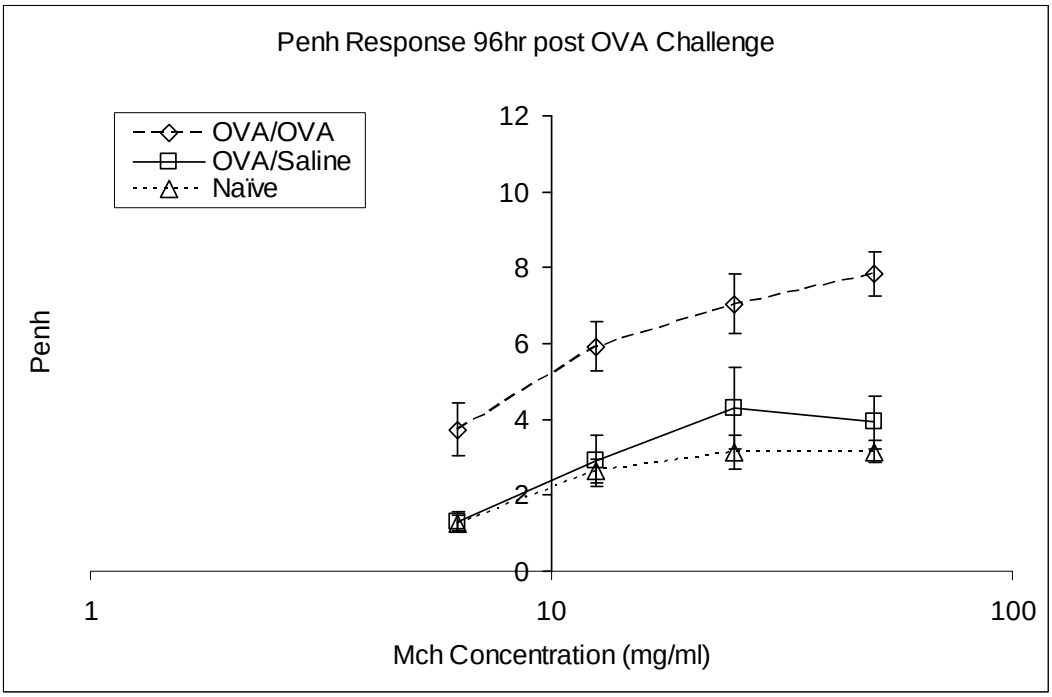

Fig. 7. Effect of Mch on Penh response on OVA model (96 hrs post dose)

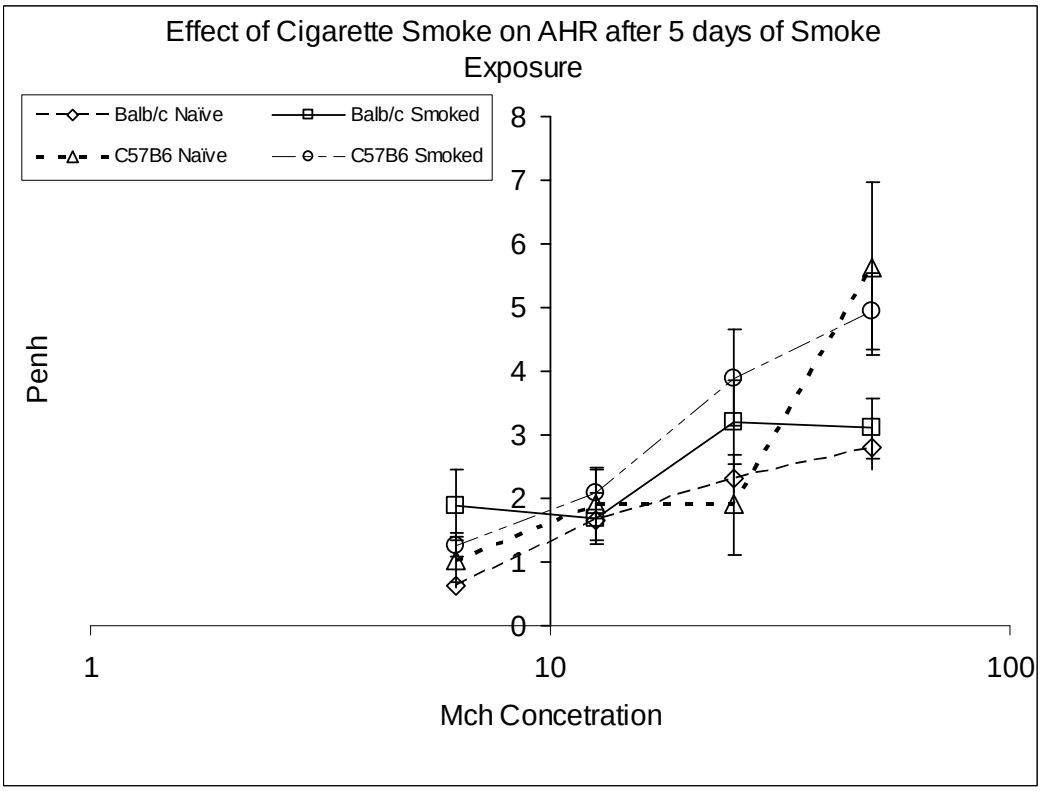

Fig. 8. Effect of Mch on Penh response on CS model (5 days of CS exposure)

3.3 Cigarette Smoke Model

In the Cigarette Smoke induced airway inflammation model (CS) evaluation, the Penh response was not a sensitive end point for the BALBc and C57Bl/6 mice. The Penh response to low concentrations of Mch was weak. The BALBc mice were consistent weak responders. For the C57Bl/6 mice there was no difference between naïve and smoke exposed immediately after 9 smoke exposures. The Penh response of the C57Bl/6 began to increase 24 hours post 9 smoke exposures. An example of the Effect of Mch on Penh response on CS model (5 days of CS exposure) is illustrated in (see above Fig. 8). The window for the Penh response reached a maximum at 8 days post last exposure. Even though the BALBc mice are non-responders, historical data shows a strong cellular response in this strain, indicating a disconnection between the cellular and the AHR components.

The tidal volume, respiratory rate and other pulmonary parameters were affected by the CS. However, these changes were not reflected by the Penh read out and more studies are needed to understand the specificity of this particular model. Based on the results from our studies, we conclude that Penh is a very robust measurement for both the LPS- and OVA-induced lung inflammation murine models which is consistent with the findings of other researchers [23]. Furthermore, our work demonstrates that the Penh response is model-dependent and it can still provide a valuable secondary readout for both LPS and OVA induced airway inflammation and hypersensitivity models.

4. CONCLUSION

In pulmonary drug discovery, the ability to quickly evaluate a drug candidate's in vivo effect is a major advantage. In this study, we evaluated Penh as a measurement in three disease relevant in vivo models in mice. We found that the optimal Mch and Penh cut off is dependent on the severity of the disease of the specific animals, model, and study. Furthermore, t the duration and intensity of the airway hypersensitivity differed by animal model. In the LPS-induced airway inflammation model, the Penh response of the LPS challenged mice was significantly elevated above that of the naïve animals at 4 hours post challenge. In the OVA induced airway inflammation model, the Penh response was up regulated 24 hours post OVA aerosol challenge, and remain elevated for 96 hours. In the CS induced airway inflammation model, the elevation of the Penh response was not optimal to provide a large enough window to observe a potential pharmacological effect. Thus, airway hypersensitivity is not a suitable endpoint for this model and more studies are needed to understand the specificity of this particular model.

Our studies successfully demonstrated that Penh is a suitable endpoint for both the LPS and OVA induced airway inflammation models. This endpoint can be easily accessed during any phase of the *in vivo* experiment and provides continue data monitoring throughout the experiment. We provide evidence that Penh can be a useful secondary complementary endpoint for measuring functional airway changes in response to inflammation. More research in this area is needed to further investigate the utility of this technology for screening drug candidates and predicting clinical efficacy of compounds.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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