# Inhalation Toxicology of Sulphur Mustard: Dose Ranging Study in a Porcine Model

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## Summary

Sulphur mustard (HD) is a chemical warfare (CW) agent with well established toxic properties. The Iran-Iraq (1980-1988) conflict demonstrated that HD is still a major CW hazard on the modern battlefield. Though known for its skin blistering properties, most of the mortality associated with HD is due to lung damage and associated infections. The aim of this work is to establish a method of exposing the lower respiratory tract to HD in an anaesthetised pig which would model humans breathing through their mouths and bypassing the nasal passages. The effects of such exposure on the physiology, biochemistry, haematology and pathology would then be investigated. The results obtained will be used to identify potential targets for therapeutic intervention.

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## 1.0 Introduction

Human deaths associated with mustard gas exposure occurred during WW1 [1], and more recently the Iran-Iraq conflict (1980-1988) demonstrated that it is still a major CW hazard on the battlefield. HD is best known as a blistering agent, however it can cause death in several ways and the majority of deaths associated with HD exposure involve some degree of pulmonary damage. Respiratory effects have been found in humans following acute and chronic exposures. In man the acute respiratory effects reported are shortness of breath and early respiratory manifestations including haemorrhagic inflammation of the tracheo-bronchial mucosa accompanied by severe erosions or membranous lesions. Progressive deterioration of lung compliance and gas exchange with resulting hypoxemia and hypercapnia were noted. More chronically, symptoms including shortness of breath, chest tightness, cough, sneezing, rhinorrhea and sore throat have been reported. Long term effects included bronchiectasis, bronchiolitis and bronchitis [2].

The recorded studies in animals of HD toxicity by inhalation are subtly different. All the animal species tested differ from man in that they have a much greater reliance on nasal breathing; indeed some are obligate nasal breathers and are unable to breath through their mouths except in extremis. For such animals the damage to the

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upper respiratory tract is more extensive and with greater effect on the animal's respiratory function than it is in man. Comparison of the results of animal exposures to those of the limited human reports show that in man there is more damage to the lower respiratory tract. This fact has prevented the observation of pathology similar to that seen in man using animal models.

The pig was chosen for these investigations because the physiology and physiological defence mechanisms are similar to those seen in man [3]. The application of invasive monitoring techniques and the taking of large numbers of blood samples is difficult in small animals but simple in large animals. The anatomy of the pig lung is more similar to man, than other smaller laboratory animals. Particularly important is the number of divisions of the bronchial tree present between the trachea and alveolus. Therefore extrapolation of the effects of HD from the pig to man is easier than extrapolating from rat to man [4]. At Dstl there is extensive knowledge and expertise in the use of this species in terminally anaesthetised animal studies [5].

The aim of this study was to develop the technologies associated with exposure of terminally anaesthetised pigs to HD by inhalation, bypassing the nasopharynx and allowing the investigation of the effects of inhaled HD on the deep lung. Initially a dose ranging study was performed allowing us to investigate a range of lung injuries to 6 hours post-exposure. The results from this study are presented here. Using the data derived from these experiments it is then intended to more fully investigate changes in pathophysiology which may occur up to 12 hours following exposure. This model will then be used in the development of treatment strategies for HD induced lung injury.

## 2.0 Methods

### 2.1 Surgery

Juvenile female large white pigs (mean weight 50 kg) were used. They were fed on standard pig diet and housed singularly in a purpose built animal holding facility for 7 days prior to the start of the experiment. Animals were allowed access to food until 12 hrs prior to the study period and water intake was not restricted. Animals were premedicated with intramuscular midazolam hydrochloride ("Hypnovel") and anaesthesia was induced by inhalation of Isofuorane in oxygen and nitrous oxide. Animals were intubated with a cuffed polytetrafluoroethylene (PTFE)-lined endotracheal tube and inhalational anaesthesia maintained until venous access had been obtained. Animals were maintained supine and were allowed to breathe spontaneously. Under aseptic conditions, the cervical vessels were surgically exposed and a catheter was introduced into the left jugular vein for administration of iv anaesthetics and fluid replacement. In the right internal jugular vein a multilumen central venous catheter was inserted for continuous measurement of central venous pressure and core temperature along side serial blood sampling. The left common carotid artery was canulated to enable measurement of serial systemic arterial blood pressures and the taking of samples. Femoral artery access was achieved for the measurement of cardiovascular parameters using pulse contour cardiac output (PiCCO) technologies. A Teflon suprapubic drainage catheter (Bonanno, Becton Dickinson) was introduced into the bladder via an open cystotomy. Three standard ECG electrodes were attached to the skin to allow recording of the electrocardiogram. Insensible fluid losses were corrected by intravenous infusion of 0.9% glucose - sodium chloride solution at a dose rate of 2.5 $ml.kg^{-1}.hr^{-1}$. Anaesthesia was subsequently maintained throughout the experiment by intravenous infusion of Propofol with Alfentanil (Rapifen). Following the completion of surgery, the animal was transferred to the exposure facility where baseline measurements were taken for 1hour. A pulse oximeter was attached for peripheral oxygenation status.

## 2.2 Exposure

Sulphur mustard vapour was generated from two characterised Porton vapour generators in parallel. These are water-jacketed glass flasks, the temperature of which can be adjusted by changing the temperature of the circulating water, thus changing the output of mustard vapour. The vapour was passed either into a charcoal bed, or by switching a tap, into a carrier flow of 80 $L.min^{-1}$ passing down a length (approx 1.75 metres) of 3" diameter glass tubing, the exposure tube. All tubing upstream of the exposure tube was warmed using electrothermal heating tapes to prevent mustard condensing onto the internal walls.

The anaesthetised animal was connected to approximately the mid point of the exposure tube via the PTFE-lined ET tube, a size 2 Fleisch pneumotachograph and a sample port. Mustard vapour concentrations up- and downstream of the animal were obtained by sampling at a constant rate on "porapak" absorption tubes, which were then analysed by solvent elution and quantitative GC-FPD. In addition, real time estimates of mustard vapour concentration could be determined during exposure using a photo-ionisation detector. The pneumotachograph, combined with placement of an oesophageal pressure transducer, appropriate software (eDacq) and a laptop computer, permitted the on-line measurement of lung function indices (Figure 1, Table 1).

Animals were exposed to air or HD for up to 10 minutes, depending on the respiratory parameters of the animal and the dose to be achieved. Following exposure the animal was monitored half-hourly for physiological and cardiovascular indices for up to 6 hours post exposure (Table 2). Hourly blood and urine samples were taken for analysis, haematology and sample preparation for future cytokine analysis. Cardiac output and extravascular lung water measurements were taken at 2 hourly intervals.

| Measurement | Parameters |
|---|---|
| Edacq (EMMS Data ACQuistion) a PC based system for real-time analysis of respiratory waveforms. | Tidal volume, inspiration time, expiration time, peak inspiratory flow, peak expiratory flow, frequency, minute volume, relaxation time, end inspiratory pause, end expiratory pause, resistance, compliance, conductance and developed pressure. |

**Table 1: Edacq measurements of lung function indices.**

| Measurement | Parameters |
|---|---|
| Vital signs | Core temperature<br>systemic arterial pressure<br>Central venous pressure<br>Pulse oximetry |
| Cardiac physiology - pulse contour cardiac output monitor (PiCCO) | CONTINUOUS MEASUREMENTS:<br>Heart rate<br>Pulse Contour Cardiac output<br>Stroke volume, stroke volume variation<br>Systemic vascular resistance<br>Pulse pressure variation<br>dP max (maximum pressure velocity increase)<br>THEMODILUTION MEASUREMENTS:<br>Cardiac output<br>Cardiac function index<br>Global end-diastolic volume<br>Intrathoracic blood volume<br>Extravascular lung water<br>Pulmonary vascular permeability index |
| Blood Chemistry (arterial and venous) | pH, $pO_2$, $pCO_2$, $HCO_3$, $TCO_2$, Base excess<br>Haematocrit, $O_2$ saturation<br>Mixed venous $O_2$ saturation |
| Derived cardiovascular variables | Systemic vascular resistance (indexed)<br>Left ventricular stroke work (indexed) |
| Derived $O_2$ transport variables | Oxygen delivery and consumption<br>Arterial & mixed venous $O_2$ content<br>Shunt fraction<br>Oxygen extraction ratio |
| Urinary HD adduct analysis [6] | mono-sulphoxide β lyase metabolite |

**Table 2: Physiological and cardiovascular monitoring.**

## 2.3 Lavage

At 6 hours post-exposure the animal was killed by an intravenous overdose of sodium pentabarbitone (200 $mg.ml^{-1}$ "Euthatal") and the heart and lungs removed en block. The lavage procedure involves the placement of the fiberoptic bronchoscope down the animal's endotracheal tube, viewing the direction of travel with the integral optical lens through the tissue. The scope can then be accurately lodged in the right middle bronchus. Normal (0.9%) saline (4 x 40mL) was administered via a plastic syringe down the scope suction port, and the resultant lavage fluid withdrawn at constant pressure back into the syringe. The resultant lavage fluid was stored in a fridge (4°C) prior to analysis for soluble and cellular components.

## 2.4 Post Mortem and Histopathology

Following the lavage procedure a full post mortem examination was carried out with samples from each of the lobes, together with samples from other major organs taken, fixed in neutral buffered formalin and processed for histopathological examination using routine methods. Lung wet:dry weight ratios were determined for each lobe following drying to constant weight.

## 2.5 Statistics

The data from each dose group has been analysed and the data expressed as mean ± standard error (se). Statistical analysis was performed as follows on individual animal data:
The area under the curve was derived for each animal and the following time periods analysed:

0 - 3hrs
3 - 6hrs

A 2-tailed t-test was performed using the individual animal data. The data compares each HD dose group to air controls.

# 3.0 Results

The data presented in this dose ranging study compares air exposed control animals with 3 HD exposed groups (Low, Medium and High inhaled dose)(Table 3). A single animal exposed to 282 $\mu g.kg^{-1}$ is not included.

| Group | Target Inhaled Dose ($\mu g.kg^{-1}$) | Actual Inhaled Dose ($\mu g.kg^{-1}$) | Animal N° | Mortality |
|---|---|---|---|---|
| Control | | | 4 | 0/4 |
| Low | 60 | 67.2 | 5 | 0/5 |
| Medium | 100 | 100.6 | 5 | 0/5 |
| High | 150 | 157.8 | 5 | 2/5 |

**Table 3: Achieved versus target inhaled doses of HD vapour.**

The results may be summarised as follows:

- All animals exposed to air were maintained breathing spontaneously for 6 hours post exposure.
- Two animals exposed to the high dose of HD died before 6 hours post exposure.
- Measures of oxygenation (arterial oxygen tension Figure 2a) (arterial oxygen content Figure 2b) showed dose dependant decreases from 4-6 hours post-exposure.
- Respiratory acidosis occurred at higher doses of HD from 4-6 hours post-exposure (Figure 3).
- Haematological indices of peripheral RBC count, haemoglobin and haematocrit increased in a dose-dependant manner from 4-6 hours post exposure (Figure 4).
- Terminal 6 hour lavage fluid cellular analysis showed increased numbers of neutrophils in the lung in the high dose group (Figure 5).
- Terminal lavage fluid cytokine analysis showed dose-dependent increases in IL-1β, IL-8 and $TxB_2$ (Figure 6). IL-6, TNF-α, $LTB_4$ and MMP-2 were also analysed in lavage fluid but no changes seen at the 6 hour time point.
- The pathology observed was similar to that observed in human exposures with haemorrhagic inflammation of the tracheo-bronchial mucosa accompanied by severe erosions or membranous lesions (Figure 7). No evidence of frank intra-alveolar oedema was observed at this time.
- Late decreases in compliance and increased resistance were seen in three animals (two medium, one high dose animal). It is likely that six hours is too early to see major changes in respiratory parameters.
- Urinary metabolite analysis revealed the presence of the mono-sulphoxide β-lyase metabolite from 2 hours post exposure.
- No changes were observed in shunt fraction, alveolar oxygen concentration, oxygen extraction ratio and oxygen delivery or consumption.
- No changes were observed in cardiovascular parameters.
- The lung wet weight / dry weight ratios and extravascular lung water remained unchanged with any of the doses of HD.

## 4.0 Figures

In all the graphical data presented the following symbols are used to identify the different groups.

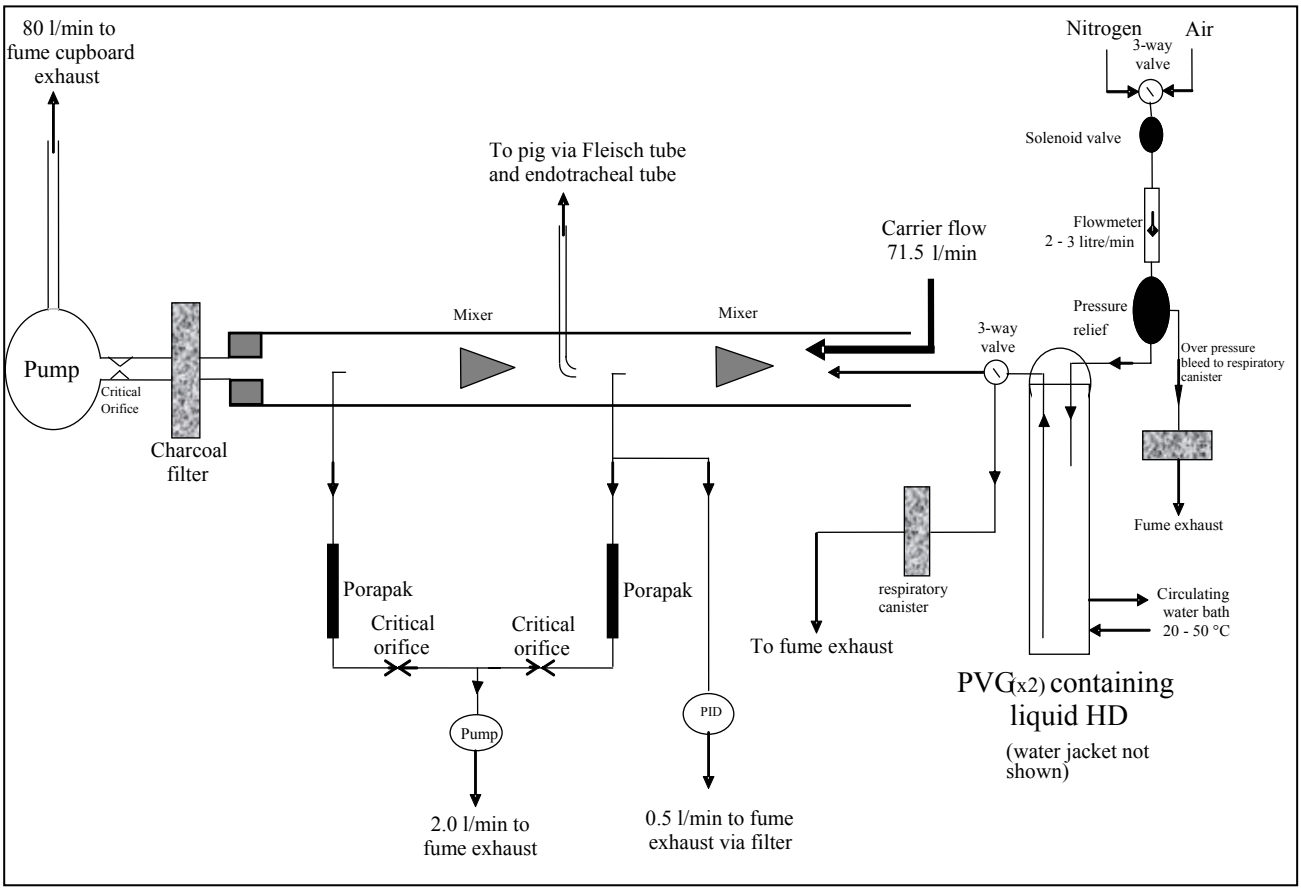



**Figure 1. Schematic showing exposure apparatus set up.**

**Figure 2: Oxygenation**

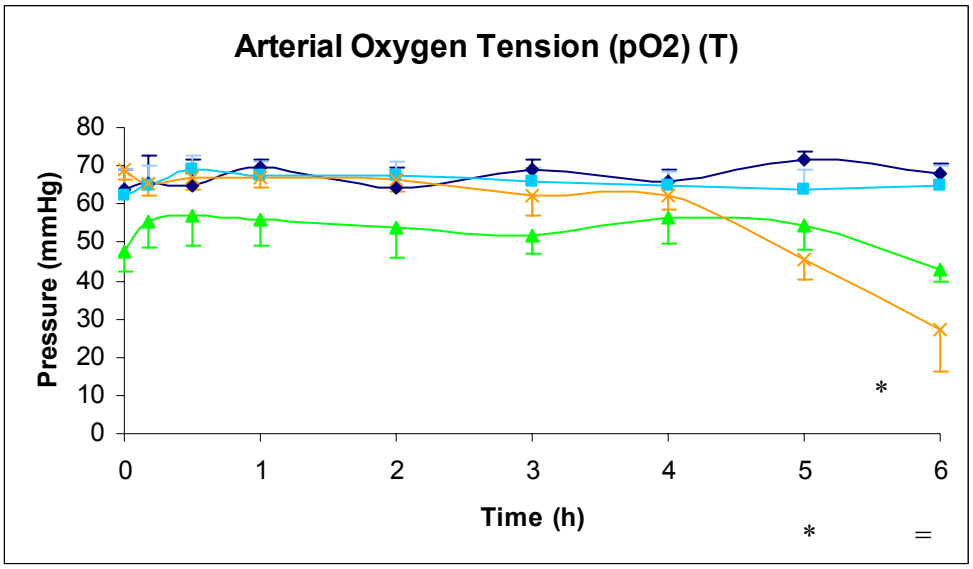



**Figure 2a: The graph above shows a dose dependant decrease in arterial oxygen tension ($pO_2$) which was most apparent 5 - 6 hours following exposure to both medium and high doses of HD.**

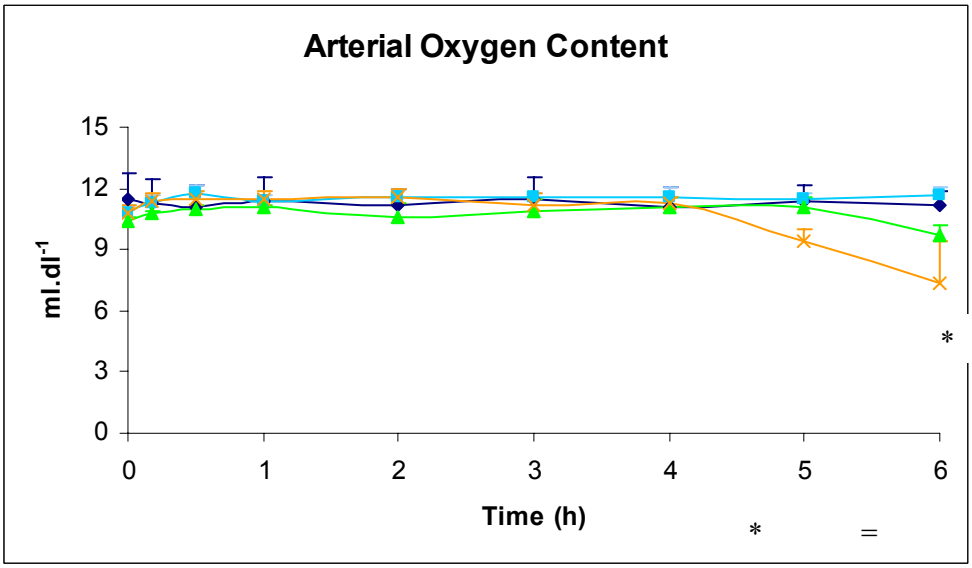



**Figure 2b: Derived Parameters**

**The reduction in oxygenation seen was reflected in the arterial oxygen content, which was reduced at 6 hours following exposure to the medium and high doses.**

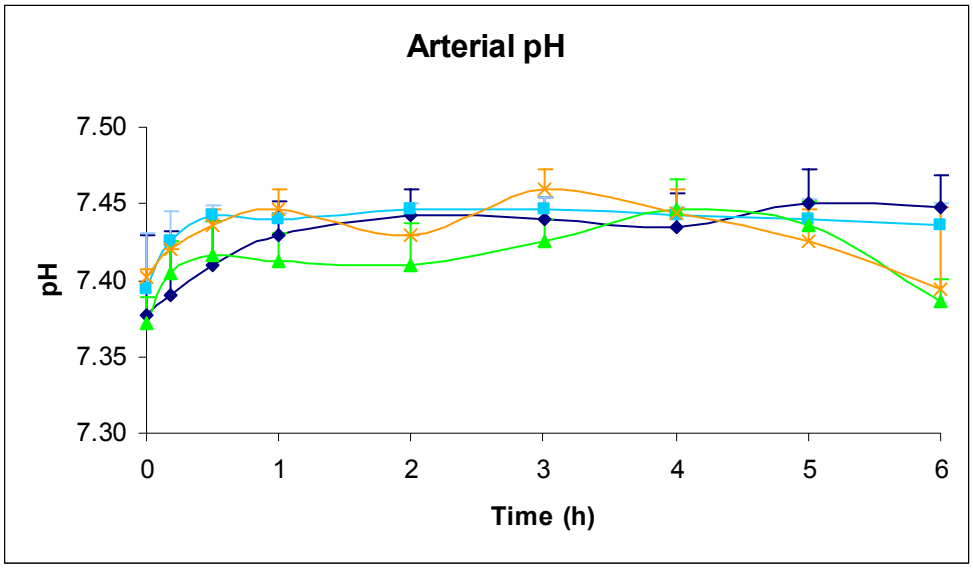
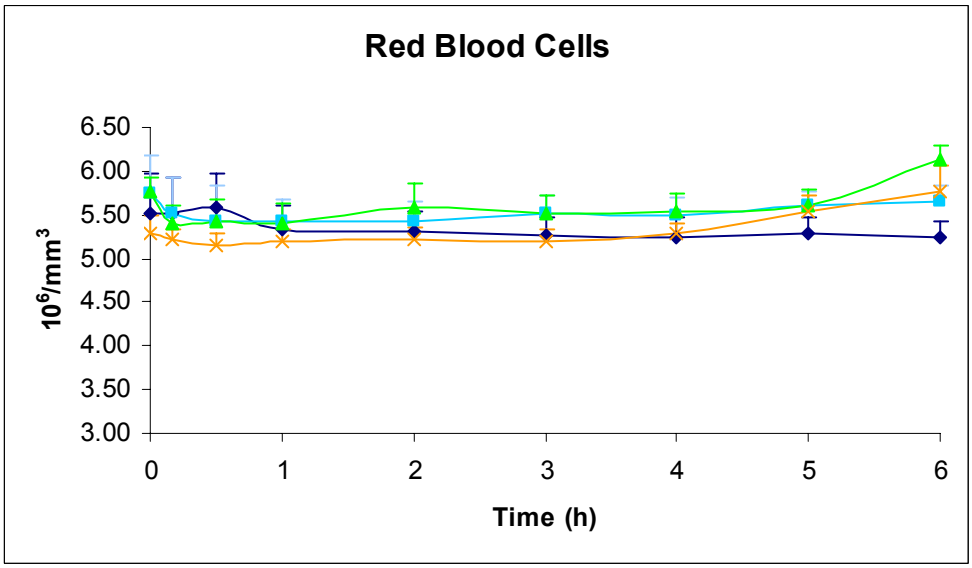



**Figure 3: Arterial pH**

**Acidosis, reflected by changes in pH and base excess were seen in both medium and high dose groups from 4 hours post exposure.**



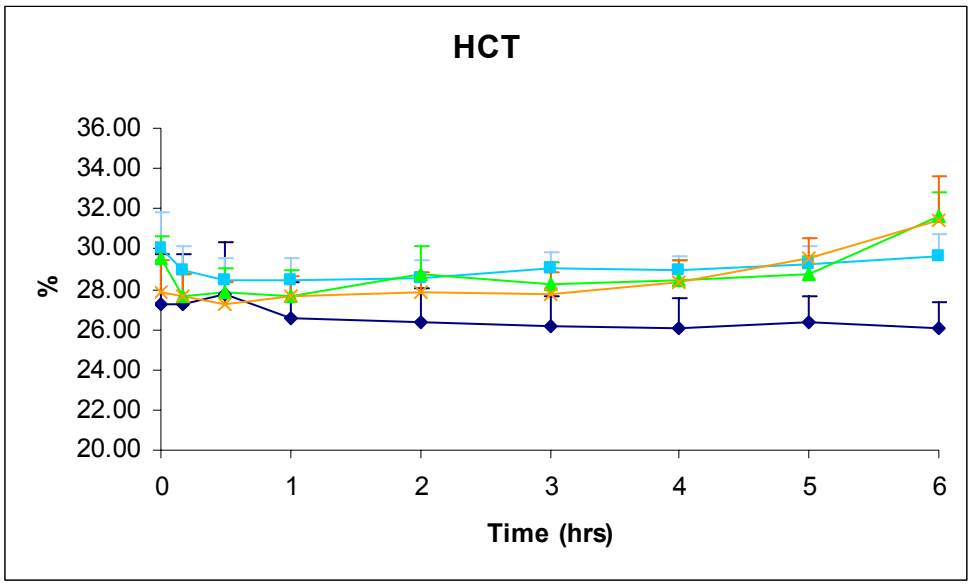



**Figure 4: Haematology**

**There was a dose related increase in RBC count, haemoglobin and haematocrit, from 4 hours post-exposure.**

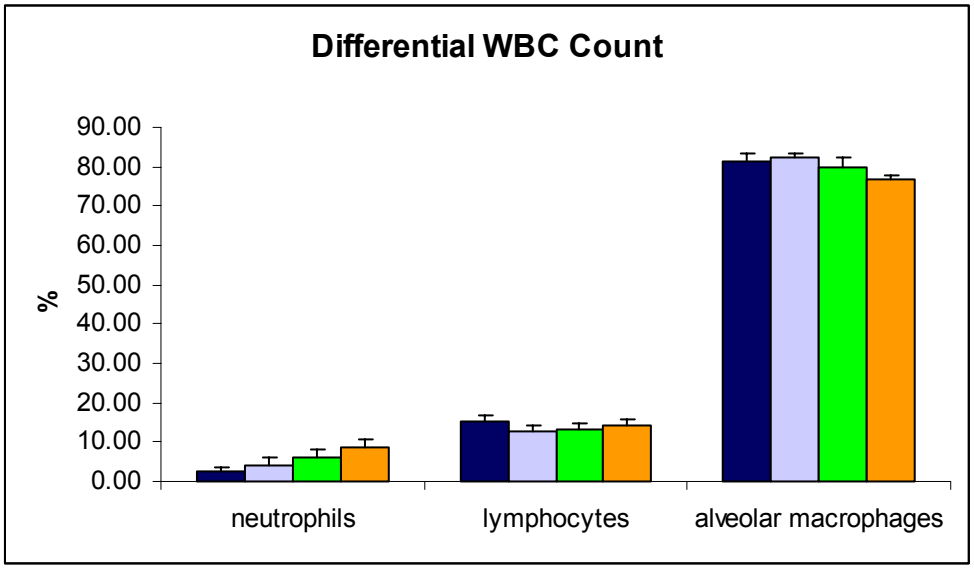



**Figure 5: Lavage fluid cellular analysis**

**There were increased neutrophil numbers in the lungs from terminal lavage fluid samples. Lymphocyte and alveolar macrophage numbers were concomitantly decreased.**

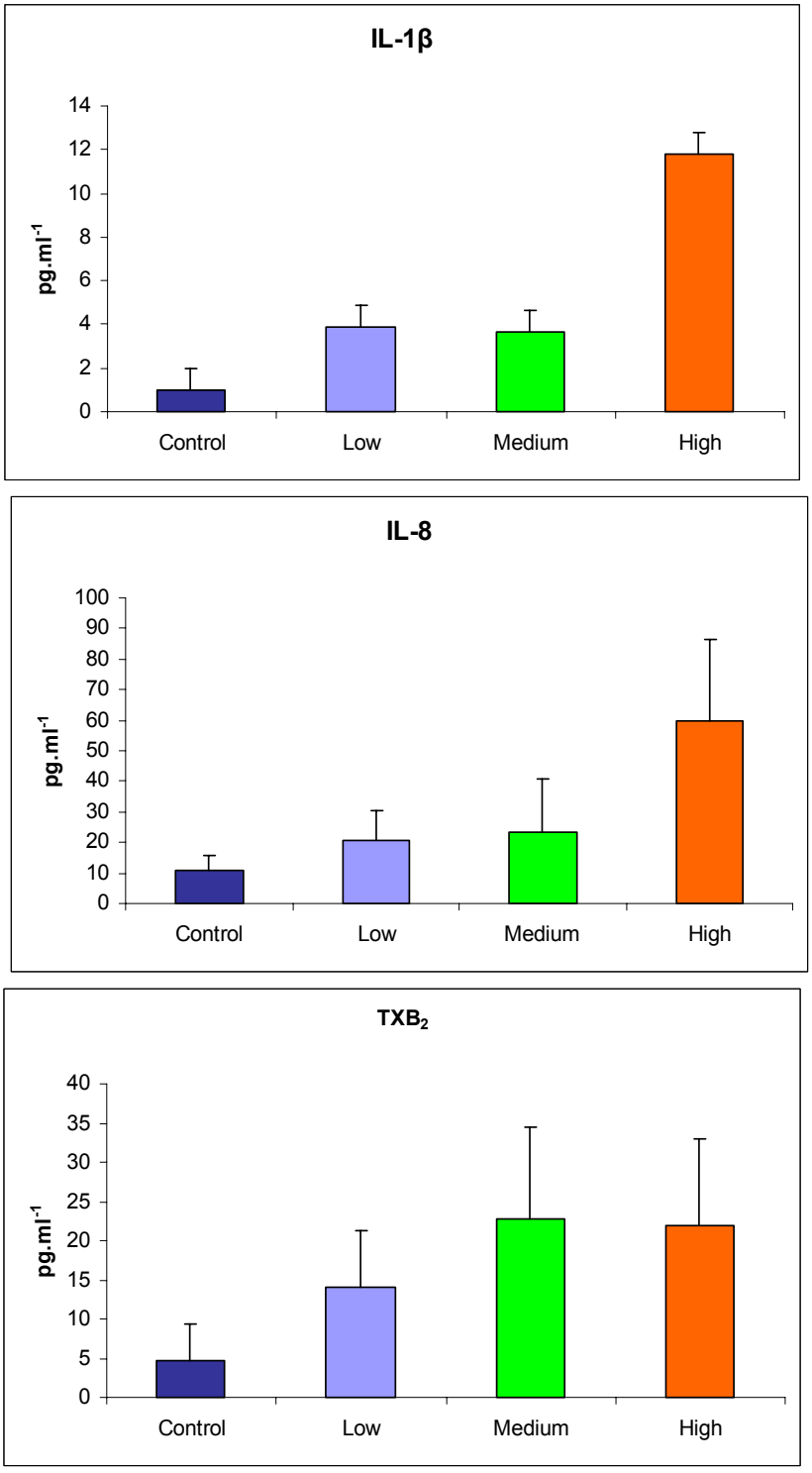
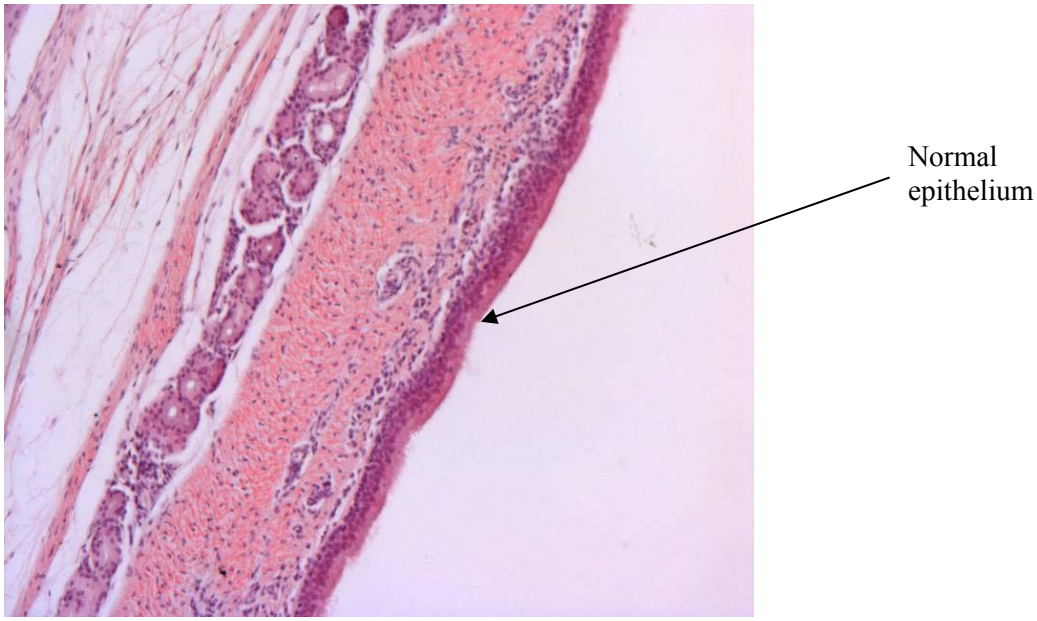



**Figure 6: Lavage fluid cytokine analysis**
**Results shown above are from analysis of bronchoalveolar lavage fluid and show dose dependant increases in IL-1β, IL-8 and $TXB_2$.**

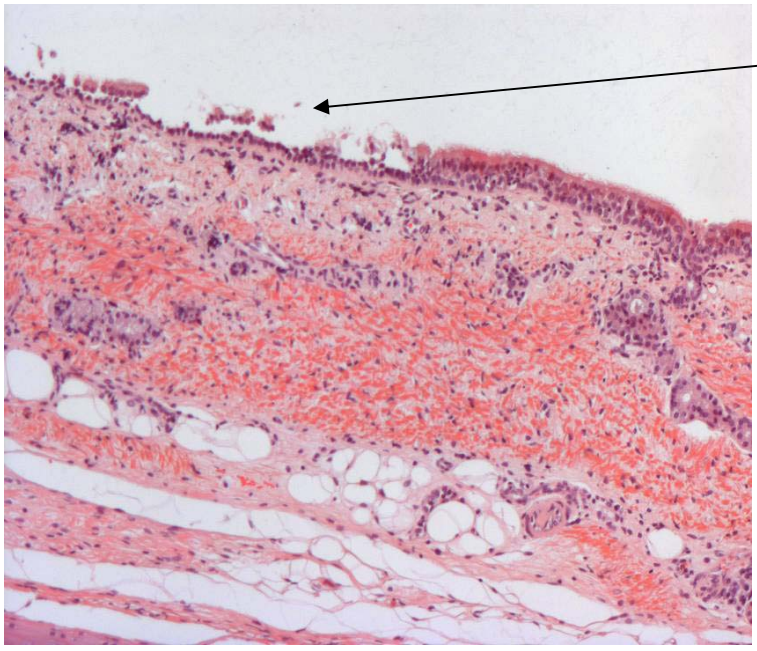
**Figure 7: Light microscopy**

The image below is of a transverse section through the trachea of an air exposed control, 6 hrs post exposure. There is normal epithelium covering the surface of the area just below the tip of the endotracheal (ET) tube.

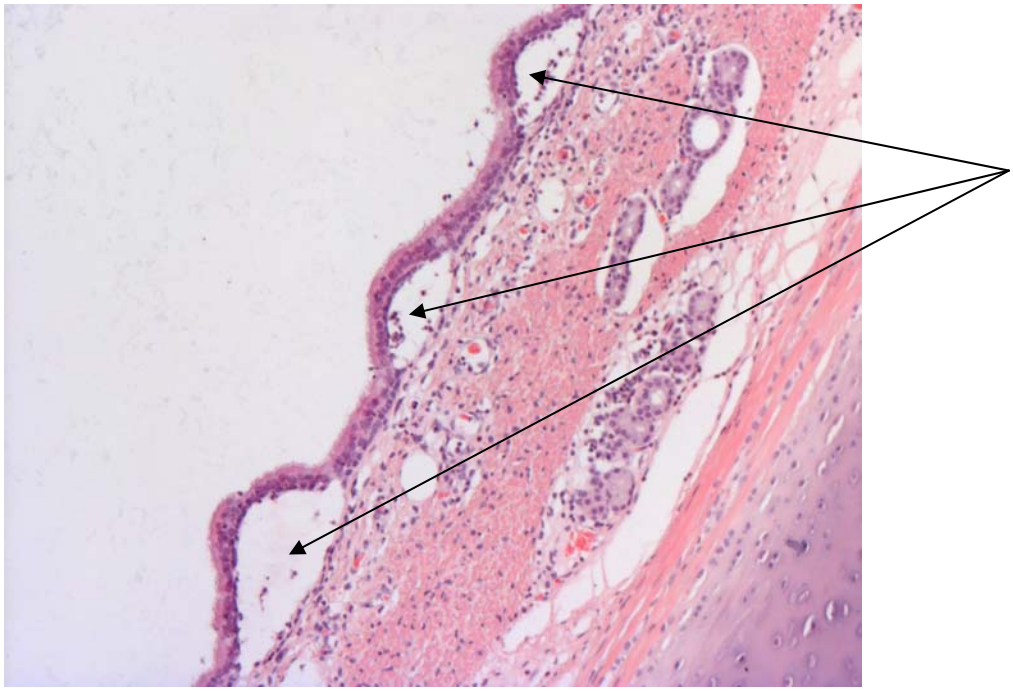



In all HD exposed animals there was evidence of epithelial necrosis sometimes associated with underlying interstitial oedema and haemorrhage in the region just below the end of the ET tube.

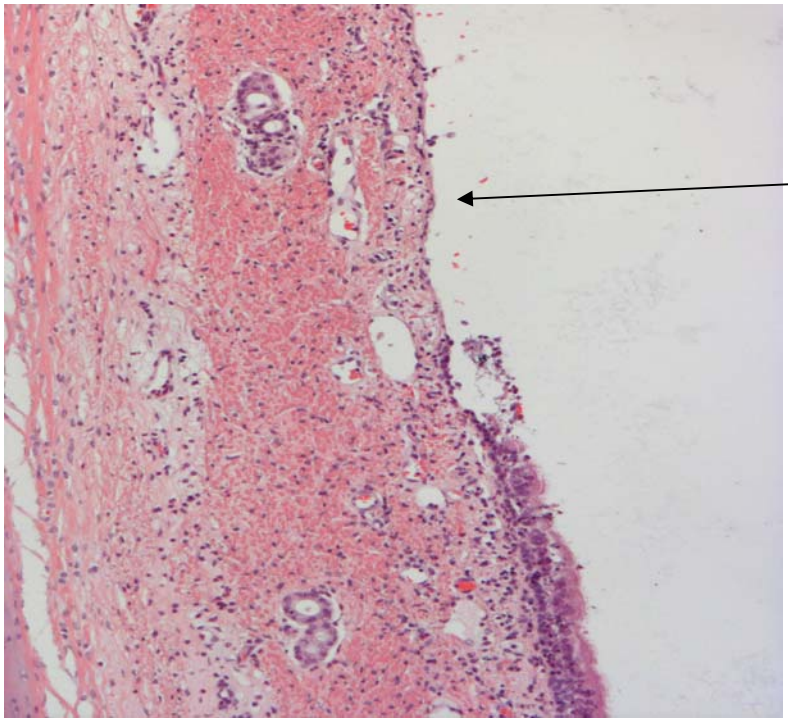
The image below is an image of a transverse section through the trachea of an animal exposed to a low dose of HD, 6 hrs post exposure. There are areas of epithelial sloughing with some necrosis of epithelial cells seen just below the tip of the ET tube.




The image below is a transverse section through the trachea of an animal exposed to a medium dose of HD, 6 hrs post exposure. There are areas showing microvesicle formation of the tracheal epithelium just below the tip of the ET tube.



The image below is a transverse section through the trachea of an animal exposed to a high dose of HD, which died 5.5 hrs post exposure. There are areas where complete sloughing of the tracheal epithelium has occurred just below the tip of the ET tube.




## 4.0 Conclusions

A reproducible model of HD-induced lung injury using an anaesthetised pig has been developed. Using this model we have been able to follow changes in cardiovascular and pulmonary dynamics for up to 6 hours following exposure in spontaneously breathing animals. During this time we have shown that the air exposed control animals have good physiology throughout the 6 hour experiment, while lying supine and spontaneously breathing, showing that the model works well. We have also shown evidence of the early stages of lung injury (decreased $O_2$ saturation) from 4 hours post-exposure in HD exposed animals. Histopathology of the lungs confirmed haemorrhagic inflammation of the tracheo-bronchial mucosa accompanied by severe erosions or membranous lesions as well as progressive deterioration of gas exchange with resulting hypoxemia and respiratory acidosis. These changes are consistent with symptoms and pathology reported in human casualties of HD exposure and allow confidence in this model for our future studies examining the time course of HD injury out to 12 hours post exposure leading on to examination of therapeutic interventions.

## 5.0 References

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