

Furosemide in the Treatment of Phosgene Induced Acute Lung Injury

C Grainge^{1,2}, AJ Smith¹, BJ Jugg¹, SJ Fairhall^{1,2}, T Mann¹, R Perrott¹, J Jenner¹, T Millar³, P Rice¹

¹ Biomedical Sciences Department, Defence Science and Technology Laboratory, Porton Down, Salisbury, Wiltshire;

² Royal Centre for Defence Medicine, Birmingham; ³ University of Southampton.

Abstract

Method: Using previously validated methods, 16 anaesthetised large white pigs were exposed to phosgene (target inhaled dose 0.3 mg kg⁻¹), established on mechanical ventilation and randomised to treatment with either nebulised furosemide (4 ml of 10 mg.ml⁻¹ solution) or saline control. Treatments were given at 1, 3, 5, 7, 9, 12, 16 and 20 hours post phosgene exposure; the animals were monitored to 24 hours following phosgene exposure.

Results: Furosemide treatment had no effect on survival, and had a deleterious effect on PaO₂: FiO₂ ratio between 19 and 24 hours. All other measures investigated were unaffected by treatment.

Conclusion: Nebulised furosemide treatment following phosgene induced acute lung injury does not improve survival and worsens PaO₂: FiO₂ ratio. Nebulised furosemide should be avoided following phosgene exposure.

Introduction

Phosgene is an industrially useful but highly poisonous gas that was previously used as a chemical warfare agent. It is currently used as an intermediate in large-scale production of a wide variety of materials including pharmaceuticals and pesticides [1]. When used as a chemical weapon in the First World War, phosgene was reportedly the most lethal of all the chemicals used, superseding chlorine due to its increased lethality [2,3]. The large quantities of phosgene produced means that there is the potential for widespread accidental or intentional exposure [4]. Release into a densely populated urban area would likely result in mass casualties and in local health care provision being overwhelmed; in such circumstances there is a requirement for effective, evidence based treatment guidelines which currently do not exist.

The effects of inhalation of phosgene gas have been well known since the beginning of the last century. Briefly, the clinical course usually consists of a minimally symptomatic exposure, followed by a completely asymptomatic latent phase that varies in duration with inhaled dose [1,5,6]. The latent phase is terminated by the onset of non-cardiogenic pulmonary oedema, usually within 24 hours of exposure; in many patients the pulmonary oedema leads to respiratory failure and death. There is currently no specific therapy for phosgene inhalation and treatment is usually based on supportive measures guided by animal studies [7-9]. Although these studies have somewhat improved our understanding of the treatment of the injury induced by inhaled phosgene, major deficiencies remain in our understanding of the underlying pathophysiology, limiting rational approaches to improving treatment.

Previously published work by our group has demonstrated, in a reproducible large animal model of phosgene induced acute lung injury, benefit to survival from invasive ventilation using a lung protective strategy, or by raising the concentration of inspired oxygen following phosgene inhalation [7,8,10]. Inhaled salbutamol or inhaled or intravenous corticosteroids had no beneficial effect on survival, though these treatments did improve some markers of injury severity [9,11]. Invasive ventilation requires full critical care facilities and these would likely be overwhelmed following a large-scale chemical release; oxygen therapy, though improving survival significantly probably does not address the underlying lung injury. There remains a requirement for an effective treatment for acute lung injury induced by phosgene inhalation which can be administered easily, is widely available and addresses and improves the underlying lung injury.

The drug furosemide is most commonly used in medicine as an oral or intravenous diuretic acting at the Na⁺/K⁺/2Cl⁻ co-transporter systems in the ascending limb of the loop of Henle in the renal tubules. For many years it has been known that furosemide has direct effects on the lung following inhaled, but not oral administration; bumetanide, which also acts at the Na⁺/K⁺/2Cl⁻ co-transporter, does not have the effects of furosemide in the lung, and it is likely that furosemide acts via other mechanisms when given by the inhaled route. [12,13].

In vitro work has shown that furosemide decreases levels of leukotriene E₄ (LTE₄), histamine, tumour necrosis factor alpha (TNF α) interleukin (IL) 6 and IL-8 as well as thromboxane (TX), probably by preventing mast cell degranulation. [14-19]. In addition to effects on airway mast cells, furosemide also inhibits lung sensory nerve stimuli and tachykinin release as well as reducing airway responses to neurokinins and inhibiting pulmonary irritant receptors [20-23]. Furosemide also decreases airway mucosal permeability and blocks the eosinophil respiratory burst [24-26]. It may also act as an antioxidant [27,28].

Corresponding author: Mr Adam Smith, Biomedical Sciences Department, Dstl Porton Down, Salisbury SP4 OJQ, UK.

Tel: +44 1980 614695 Fax: +44 1980 613741

Email: ajsmithg@dstl.gov.uk

Although the exact pathophysiological injury following phosgene inhalation is unknown, the non-cardiogenic pulmonary oedema seen is due to an increase in alveolar permeability following an oxidative reaction with organic molecules in the lung tissue; this reaction also results in the release of LTC₄, D₄ and E₄, an increase in vascular permeability and neutrophil recruitment [1]. In light of the direct lung effects of furosemide, we postulated that furosemide may act as an antioxidant, and that treatment with inhaled furosemide following phosgene induced acute lung injury might be beneficial.

In order to further elucidate the underlying injury following phosgene inhalation over time, we took samples via a bronchoscope for later RNA analysis at various time points before and after inhalation.

Here we demonstrate that, despite furosemide having potent antioxidant properties, when used alone it confers no discernible benefits following phosgene inhalation above nebulised saline treatment.

Methods

Large white juvenile female pigs (47-55 Kg) (n=18) were obtained from an approved commercial source. Animals were housed in pairs and allowed access to food and water *ad libitum* for 5 days, as previously described [10]. All experiments were carried out in accordance with the Animals (Scientific Procedures) Act, 1986, and approved after ethical review at Dstl, Porton Down.

Surgical procedures

The full experimental procedure has been previously described in detail. [9].

Briefly, following pre-medication, animals were initially anaesthetised with isoflurane in 70% oxygen, intubated and then maintained on total intravenous anaesthesia using Fresenius Propoven 1% ("Propofol" Fresenius Kabi Ltd, Cheshire, UK) and Alfentanil Hydrochloride (Rapifen, Janssen Pharmaceuticals Ltd, County Cork, Ireland). Once total intravenous anaesthesia was established, animals were ventilated on room air. The left and right internal jugular veins, the left common carotid artery and the left femoral artery were catheterised following surgical exposure. A foley urinary catheter was introduced via an open cystotomy. Electrocardiogram, pulse oximetry, exhaled carbon dioxide, and central venous pressure were measured using a Propaq 106EL monitor (Protocol systems Inc., Beaverton, USA) whilst cardiac output was measured using a Pulse Contour Continuous Cardiac Output catheter (PiCCO, Pulsion Medical Systems AG, Munich, Germany).

A maintenance infusion of 0.9% sodium chloride and 4% glucose (2.5 ml kg⁻¹ hr⁻¹) was delivered to replace insensible losses.

Animals were exposed, whilst spontaneously breathing, to phosgene (target inhaled dose 0.3 mg kg⁻¹, mean achieved inhaled dose 0.266 +/- 0.00825 mg.kg⁻¹ for the control group and 0.256 +/- 0.0159 mg.kg⁻¹ for the treated group). Between 30 mins and 1 hour following exposure, anaesthesia was deepened and the animals were established on a mechanical ventilator (Evita XL, Draeger Ltd) using Intermittent Positive Pressure Ventilation (IPPV), FiO₂ 0.21, tidal volume 10 ml Kg⁻¹, frequency 20 breaths min⁻¹, positive end-expiratory pressure (PEEP) 3cm water.

Treatment regimens

Two animals were subject to a sham phosgene exposure, using atmospheric air and received no nebulised treatment. These

were used for control tissues for the RT-qPCR analysis. All other animals were exposed to inhaled phosgene. Exposed animals were randomly allocated to treatment or control groups, n=8 for each group. Treatment consisted of 4 ml of a 10 mg ml⁻¹ solution of furosemide (Antigen Pharmaceuticals LTD, Ireland) administered as an aerosol generated over 15 minutes using an Aeroneb Lab micropump nebuliser (Aerogen (Ireland) Ltd, Galway Ireland) placed in the inspiratory limb of the ventilator circuit. Treatments were given at 1, 3, 5, 7, 9, 12, 16 and 20 hours post phosgene exposure. Control group animals were treated with nebulised 0.9 % w/v saline using the same protocol.

Measurements

Physiological measurements were made every 20 minutes for a baseline of one hour. After this time, measurements were recorded every 30 minutes until the end of the experimental period (24 hours). Derived variables were calculated using standard formulae [29]. Arterial and mixed venous blood gas samples were taken at hourly intervals and immediately analysed (GEM Premier 3000, Instrumentation Laboratory). Haematological analysis was performed on EDTA blood samples using a Coulter Ac-T 5 diff CP (Beckman Coulter) series analyser. Differential peripheral white blood cell (WBC) counts were performed manually.

At the conclusion of the 24 hour observation period or when the animal became moribund (defined as asystole and central venous oxygenation of <15 %), the animal was culled by an intravenous overdose of sodium pentobarbitone (200 mg.ml⁻¹) (Euthatal, Rhone Merieux Ltd., Harlow, Essex), and a post mortem examination was performed. Bronchoalveolar lavage (BAL) of the right middle lobe was performed using 4 x 40 ml of sterile 0.9 % w/v saline. Lavage fluid was analysed for total WBC counts using a Coulter Ac-T 5 diff CP and for differential cell counts (Shandon Cytospin, 1800 rpm, 10 minutes). Slides were stained with DifQuik stain and 100 cells counted. Protein content of BAL supernatant was determined using the Coomassie blue method [30]. Remaining supernatant was stored at -80°C for subsequent analysis of IL-1, IL-6 and IL-8 using commercial porcine ELISA kits (R&D Systems, Abingdon, Oxon, UK).

Following lavage, the lungs were weighed, the weight of the remaining lavage saline taken into account, for lung wet weight / body weight determinations and lung wet weight to dry weight ratio. Samples from each lobe, and all major organs were taken, fixed in neutral buffered formalin and processed for histopathological examination using routine techniques. Histology slides were sent to the Veterinary Laboratories Agency for a qualified independent pathologist to score the slides for pathological changes. The pathologist was blinded to the study design and used a previous developed [8] scoring system to assess the lung pathology where a score of zero represented no damage and a score of three represented severe damage. A 1cm³ sample from the distal portion of the left upper lobe was also taken into RNA later (Ambion / Applied Biosystems, Warrington, UK) which prevents degradation of RNA, and stored at -20°C for subsequent RNA extraction.

Bronchoscopy

Bronchoscopy was performed according to guidelines issued by the British Thoracic Society using a flexible fiberoptic bronchoscope (Olympus BF-4B2, KeyMed Ltd, Essex) [31]. Single biopsies were taken from the third to fifth generation

carinae on the left side at time points -1, 2, 6 and 13 hours post exposure and multiple biopsies taken at 24 hours or time of death. Biopsies were stored in RNA later at -20°C for subsequent RNA extraction.

RNA extraction and RT-qPCR

The full experimental protocol is given in the online supplement (available at www.ramcjournal.com). Briefly, RNA was extracted from bronchial biopsies using the Trizol reagent kit (Invitrogen, Paisley, United Kingdom), cDNA produced and multiple genes of interest examined using reverse transcription quantitative polymerase chain reaction. Expression of genes of interest was expressed using the delta delta CT method using housekeeping genes identified using a geNorm housekeeping selection kit (PrimerDesign, Southampton, United Kingdom) and software. [32,33].

Antioxidant capacity assays

The antioxidant capacity of furosemide was measured by assays comparing two different known antioxidant mechanisms, namely hydrogen atom transfer (using the Oxygen Radical Absorbance assay (ORAC)) [34] and electron transfer (using the CUPRAC assay) [35]. Full details of these assays are given in the online supplement.

Statistical analysis

Clinical data

Clinical and exposure data was normally distributed, therefore analysis was performed using a 2-tailed Student's *t* test for individual measurements e.g. bronchoalveolar lavage differentials. Analysis of multiple measurement recordings e.g. $\text{PaO}_2:\text{FiO}_2$ ratio, was performed using area under the curve (AUC) determinations in a 2-tailed Student's *t* test for the following time points: -1 to 0, 0 to 6, 7 to 12, 13 to 18, 19 to 24 hours. Results were expressed as mean \pm (SE) and *p* values < 0.05 were considered significant.

RT-qPCR analysis

RT-qPCR data were not normally distributed and therefore analysis was performed using Kruskal Wallis for comparison between all three groups, then Mann Whitney between each of the groups (air vs furosemide, air vs phosgene only, furosemide vs phosgene) at each of the time points. Results were expressed as mean \pm (SE) and *p* values < 0.05 were considered significant.

Results

Exposure doses

The achieved mean inhaled doses were $0.266 \pm 0.00825 \text{ mg.kg}^{-1}$ for the control group and $0.256 \pm 0.0159 \text{ mg.kg}^{-1}$ for the treated group (*p*=ns).

Antioxidant capacity assay

Compared to vitamin C (100%), furosemide was a less effective antioxidant than N-acetylcysteine (NAC) by the hydrogen atom transfer mechanism (NAC $76.7 \pm 2.4\%$; furosemide $43.8 \pm 2.7\%$, mean \pm SE *n*=8 per group). The CUPRAC assay which measures antioxidant capacity by the electron transfer mechanism showed that whilst NAC had some antioxidant effect by this measure, furosemide did not (NAC $56.3 \pm 0.4\%$; furosemide $0.1 \pm 0.2\%$; mean \pm SE *n*=8 per group; Figure 1).

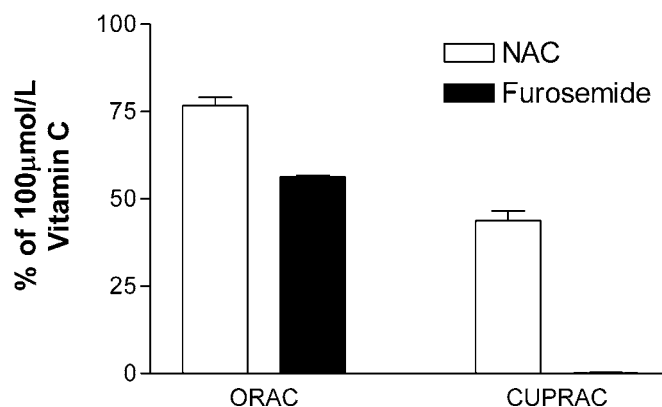


Figure 1: Antioxidant capacity of furosemide and N-acetylcysteine compared to vitamin C by ORAC and CUPRAC assays. Furosemide showed some anti-oxidant effect by the ORAC but not the CUPRAC assay. Bars are mean \pm SE; *n*=8 per group

Survival

Three animals from the phosgene control group and one from the treated group died prior to the end of the 24 hour observation period. There was no difference in survival between the furosemide and saline treated groups at 24 hours. This is illustrated as a Kaplan-Meier plot of survival over time in Figure 2.

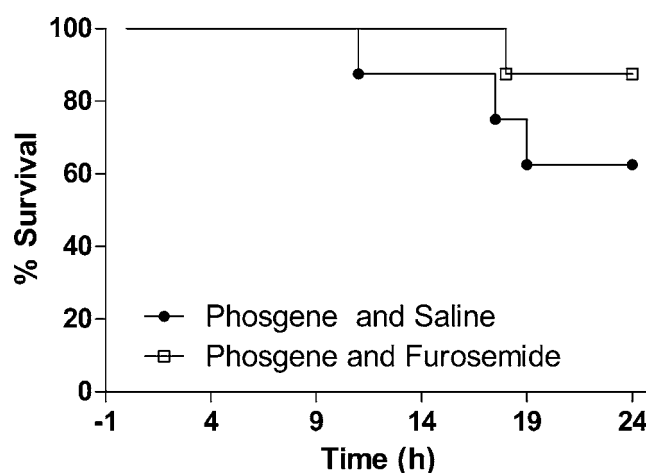


Figure 2: Percentage survival of animals exposed to phosgene (inhaled dose $0.266 \pm 0.00825 \text{ mg.kg}^{-1}$ for the control group and $0.256 \pm 0.0159 \text{ mg.kg}^{-1}$ for the treated group) and treated with either saline (4 ml 0.9% w/v) or furosemide (4 ml of a 10 mg ml^{-1} solution) given at 1,3,5,7,9,12,16 and 20 hours following phosgene exposure.

Oxygenation and shunt fraction (Q_s/Q_t)

There were no significant differences in arterial partial pressure of oxygen (PaO_2) between the control and treatment groups (data not shown). The $\text{PaO}_2:\text{FiO}_2$ ratio is a derived parameter used clinically as an index of hypoxaemia when assessing acute lung injury and acute respiratory distress syndrome. The group treated with furosemide had a statistically significantly (*p*<0.05) lower $\text{PaO}_2:\text{FiO}_2$ ratio between 19 and 24 hours (Figure 3). There were no statistically significant effects on shunt fraction ($Q_s:Q_t$) between groups (data not shown).

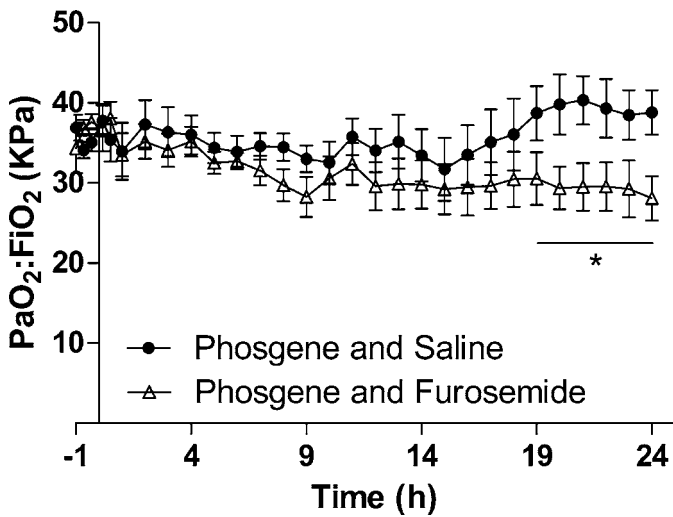


Figure 3: Changes in PaO₂ : FiO₂ ratio in animals exposed to phosgene (inhaled dose 0.266 +/- 0.00825 mg.kg⁻¹ for the control group and 0.256 +/- 0.0159 mg.kg⁻¹ for the treated group) and treated with either saline (4mls 0.9%) or furosemide (4 ml of a 10 mg ml⁻¹ solution) given at 1,3,5,7,9,12,16 and 20 hours following phosgene exposure. Furosemide significantly decreased PaO₂ : FiO₂ ratio (*p<0.05) from 19-24h. Points are means ± SE; n=8 per group.

Lung Wet Weight to Body Weight ratio, Lung Wet Weight to Dry Weight ratio and lavage fluid protein

Lung wet weight to body weight (LWW:BW) ratio and lung wet weight to dry weight (LWW:DW) ratio are measures of extravascular lung water and an indicator of the degree of alveolar permeability following acute lung injury. There was no significant difference in LWW:BW ratio or LWW:DW ratio between the two groups. There was also no difference in lavage protein concentration.

Differential White cell count.

Historically, air exposed animals show the alveolar macrophage to be the predominant cell in the alveoli with <5% neutrophils present. The phosgene control animals from this study demonstrated a phosgene induced influx of neutrophils into the alveolar space of 26%. There was no effect of furosemide on the inflammatory response within the lung as measured by differential white cell counts performed on bronchoalveolar lavage (Figure 4).

RT qPCR

There were no differences between the phosgene control and treatment groups for any of the genes of interest studied (data not shown).

Inflammatory mediators

IL-1, IL-6, IL-8 and C-reactive protein (CRP) were all measured in peripheral blood and BALF (data not shown) but there were no differences between groups treated with furosemide and saline treated controls.

Discussion

This study has shown that nebulised furosemide treatment initiated soon after inhaled phosgene exposure has no effect on survival or multiple measures of acute lung injury over 24 hours following poisoning.

Despite extensive investigation of potential treatments for phosgene induced acute lung injury, the underlying mechanism of the injury is not well understood [4]. The current working hypothesis is that an oxidant cascade is initiated following phosgene inhalation with subsequent release of multiple inflammatory mediators; this combined with a delayed neutrophil influx lead to the clinical findings of pulmonary oedema secondary to increased alveolar permeability.

Furosemide has been shown to inhibit the release of multiple inflammatory mediators within the lungs when administered by inhalation [14-17] and also decreases alveolar permeability in an experimental model [25]. By an unknown mechanism it also decreases dyspnoea associated with both experimental conditions and terminal lung disease [36]. Furosemide is readily available, widely used, cheap and safe to be administered by nebuliser [37].

Despite these previously demonstrated effects and the hypothesised beneficial effects of furosemide following phosgene induced acute lung injury, we show no benefit of regularly administering nebulised furosemide in our large animal model. This pilot study used small numbers, and though there were fewer deaths in the furosemide treatment group, the experimental numbers would have had to be increased to 37 per group to determine whether this was a real difference with 80% power, the absence of any trend towards difference in the monitoring data would suggest, we feel, that the lack of a positive result is not due to a type II error, rather the lack of efficacy of furosemide in the treatment of phosgene induced acute lung injury. N – acetylcysteine (NAC), used here in comparison to furosemide in the antioxidant assay, has previously been administered in an isolated lung preparation to rabbit lungs which had been exposed to phosgene [38]. The NAC protected against phosgene induced rises in multiple markers of cellular damage. The authors postulated that the NAC acted as an antioxidant, maintaining levels of glutathione, reducing lipid peroxidation and arachidonic acid metabolites. Similar, though less pronounced effects were found when feeding mice a diet

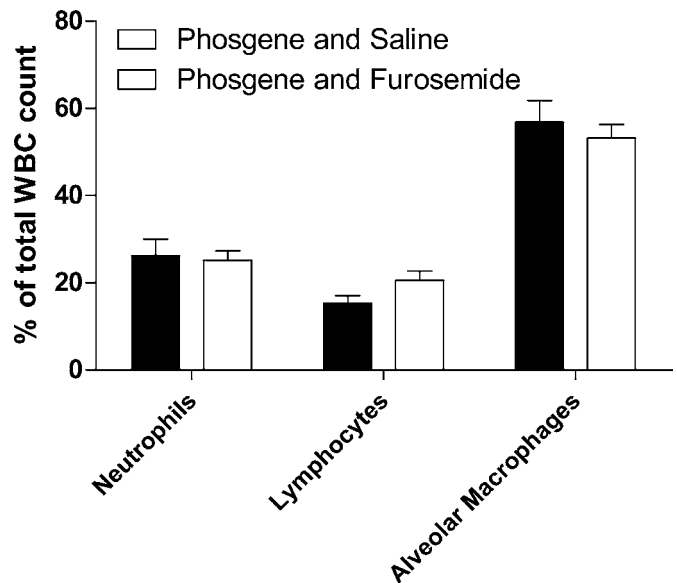


Figure 4: Differential white cell count in bronchoalveolar lavage from animals exposed to phosgene (inhaled dose 0.266 +/- 0.00825 mg.kg⁻¹ for the control group and 0.256 +/- 0.0159 mg.kg⁻¹ for the treated group) and treated with either saline (4mls 0.9%) or furosemide (4 ml of a 10 mg ml⁻¹ solution) given at 1,3,5,7,9,12,16 and 20 hours following phosgene exposure. Bars are means ± SE; n=8 per group.

rich in oral antioxidants, either butylated hydroxyanisole (BHA) or n-propyl gallate (nPG) [39,40].

Since NAC and furosemide act as antioxidants, the finding that furosemide fails to improve survival following phosgene inhalation may imply that the previous small animal and isolated lung work on NAC cannot be extrapolated to large animals. It is also possible that NAC exerts its antioxidant effects by the electron transfer pathway, which furosemide does not alter.

Our group has previously demonstrated that phosgene induced ALI is improved by delayed administration of oxygen, and that the inflammatory response is improved by regular administration of nebulised salbutamol, but at the expense of ventilation perfusion matching [8,9]. The most significant improvement in survival following phosgene inhalation is following positive pressure ventilation based on ARDSnet recommendations [7, 41].

Despite furosemide having multiple direct lung effects, and acting as an antioxidant by the hydrogen atom transfer mechanism, nebulised administration soon after phosgene inhalation results in no change in survival at 24 hours. Use of furosemide as a single treatment following acute phosgene induced lung injury is of no benefit and should be avoided.

Acknowledgements

The authors would like to thank the inhalation exposure team, the animal services staff and the histopathology team for their contribution to the study.

References

- Borak J, Diller WF. Phosgene exposure: mechanisms of injury and treatment strategies. *J Occup Environ Med* 2001; **43**:110-119
- Grainge C. Breath of life: the evolution of oxygen therapy. *J Royal Soc Med* 2004; **97**(10):489-493
- Marrs TC, Maynard RJ, Sidell FR. Phosgene. In: Marrs TC, Maynard RJ and Sidell FR (eds) *Chemical Warfare Agents: Toxicology and Treatment*. Chichester: John Wiley & Sons: 1996; Ch 8:185-202
- Sciuto AM, Hurt HH. Therapeutic treatments of phosgene-induced lung injury. *Inhal Toxicol* 2004; **16**(8):565-80
- Wyatt JP, Allister CA. Occupational phosgene poisoning – a case report and review. *J Accid Emerg Med* 1995; **12**:212-213.
- Diller WF. Pathogenesis of phosgene poisoning. *Toxicol Ind Health* 1985; **1**(2):7-15
- Parkhouse, DA, Brown RF, Jugg BJ *et al*. Protective ventilation strategies in the treatment of phosgene induced lung injury. *Mil Med* 2007; **172**(3):295-300
- Grainge C, Jugg BJ, Smith AJ *et al*. Delayed low-dose supplemental oxygen improves survival following phosgene-induced acute lung injury. *Inhal Toxicol* 2010; **22**(7):552-60
- Grainge C, Brown RF, Jugg BJ *et al*. Early treatment with nebulised salbutamol worsens physiological measures and does not improve survival following phosgene induced acute lung injury. *J R Army Med Corps* 2009; **155** (2):105-9
- Brown RFR, Jugg BJA, Harban FMJ *et al*. Pathophysiological responses following phosgene exposure in the anaesthetised pig. *J Appl Toxicol* 2002; **22**:263-269
- Smith AJ, Brown RF, Jugg BJ *et al*. The effect of steroid treatment with inhaled budesonide or intravenous methylprednisolone on phosgene-induced acute lung injury in a porcine model. *Mil Med* 2009; **174**(12):1287-94
- O'Connor BJ, Chung KF, Chen-Worsdell YM, Fuller RW, Barnes PJ. Effect of inhaled furosemide and bumetanide on adenosine 5'-monophosphate- and sodium metabisulfite-induced bronchoconstriction in asthmatic subjects. *Am Rev Respir Dis* 1991; **143**(6):1329-33
- Bianco S, Vaghi A, Robuschi M, Pasargiklian M. Prevention of exercise-induced bronchoconstriction by inhaled frusemide. *Lancet* 1988; **2**(8605):252-55
- Anderson SD, He W, Temple DM. Inhibition by furosemide of inflammatory mediators from lung fragments. *N Eng J Med* 1991; **324**(2):131
- Berti F, Rossoni G, Zuccari G *et al*. Protective activity of inhaled frusemide against immunological respiratory changes and mediator release in guinea-pigs. *Pulm Pharmacol* 1992; **5**(2):115-20
- Echazarreta AL, Gómez FP, Ribas J *et al*. Effects of inhaled furosemide on platelet-activating factor challenge in mild asthma. *Eur Respir J* 1999; **14**(3):616-21
- Yuengsrigul A, Chin TW, Nussbaum E. Immunosuppressive and cytotoxic effects of furosemide on human peripheral blood mononuclear cells. *Ann Allergy Asthma Immunol* 1999; **83**(6 Pt 1):559-66
- Redrup AC, Pearce FL. Effect of loop diuretics on rat peritoneal and human lung mast cells. *Agents Actions* 1994; **41**:C47-8
- Croce M, Costa Manso E, Gato JJ, Córdoba H, Oehling A. Furosemide inhibits in vitro histamine release induced by antigens and anti-IgE. *J Investigational Allergol & Clin Immunol : official organ of the International Association of Asthmology (INTERASMA) and Sociedad Latinoamericana de Alergia e Inmunología* 1992; **2**(4):205-10
- Rajakulasingham K, Polosa R, Church MK, Howarth PH, Holgate ST. Effect of inhaled frusemide on responses of airways to bradykinin and adenosine 5'-monophosphate in asthma. *Thorax* 1994; **49**(5):485-91
- Franova S. The influence of inhaled furosemide on adverse effects of ACE-inhibitors in airways. *Bratislavské lekárske listy* 2001; **102**(7):309-13
- Crimi N, Prosperini G, Ciamarra I, Mastruzzo C, Magri S, Polosa R. Changes in neurokinin A (NKA) airway responsiveness with inhaled frusemide in asthma. *Thorax* 1997; **52**(9):775-79
- Sudo T, Hayashi F, Nishino T. Responses of tracheobronchial receptors to inhaled furosemide in anesthetized rats. *Am J Respir Crit Care Med* 2000; **162**(3 Pt 1):971-5
- Corboz MR, Ballard ST, Inglis SK, Taylor AE. Dilatory effect of furosemide on rat tracheal arterioles and venules. *Am J Respir Crit Care Med* 1997; **156**(2 Pt 1):478-83
- Inoue T, Shigeta M, Mochizuki H *et al*. Effect of inhaled furosemide on lung clearance of technetium-99m-DTPA. *J Nucl Med* 1995; **36**(1):73-77
- Perkins RS, Dent G, Chung KF, Barnes PJ. The effect of anion transport inhibitors and extracellular Cl⁻ concentration on eosinophil respiratory burst activity. *Biochem Pharmacol* 1992; **43**(11):2480-83
- Lahet JJ, Lenfant F, Courderot-Masuyer C *et al*. In vivo and in vitro antioxidant properties of furosemide. *Life Sci* 2003; **73** (8):1075-82
- Kang MY, Tsuchiya M, Packer L, Manabe M. In vitro study on antioxidant potential of various drugs used in the perioperative period. *Acta Anaesth Scand* 1998; **42** (1):4-12
- Edwards JD, Shoemaker WC, Vincent JL. *Oxygen Transport: Principles and Practice*. London: WB Saunders, 1993.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976; **72**:248-54
- British Thoracic Society Bronchoscopy Guidelines Committee, a Subcommittee of Standards of Care Committee of British Thoracic

- Society. British Thoracic Society guidelines on diagnostic flexible bronchoscopy. *Thorax* 2001; 56(Suppl 1):i1-21
32. Vandesompele J, De Preter K, Pattyn F *et al*. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2002; **3(7)**: RESEARCH0034
33. Skrzypski M. Quantitative reverse transcriptase real-time polymerase chain reaction (qRT-PCR) in translational oncology: lung cancer perspective. *Lung Cancer* 2008; **59(2)**:147-54
34. Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 2005; **53(6)**:1841-56.
35. Apak R, Güçlü K, Ozyürek M, Karademir SE. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *J Agric Food Chem* 2004; **52(26)**:7970-81.
36. Nishino T, Ide T, Sudo T, Sato J. Inhaled furosemide greatly alleviates the sensation of experimentally induced dyspnea. *Am J Respir Crit Care Med*. 2000;**161(6)**:1963-67.
37. Yen ZS, Chen SC. Best evidence topic report. Nebulised furosemide in acute adult asthma. *Emerg Med J* 2005; 22(9):654-55
38. Sciuto AM, Strickland PT, Kennedy TP and Gurtner GH. Protective effects of N-acetylcysteine treatment after phosgene exposure in rabbits. *Am J Respir Crit Care Med* 1995;**151(3 Pt 1)**:768-72
39. Sciuto AM, Moran TS. BHA diet enhances the survival of mice exposed to phosgene: the effect of BHA on glutathione levels in the lung. *Inhal Toxicol* 1999; **11(9)**:855-71
40. Sciuto AM, Moran TS. Effect of dietary treatment with n-propyl gallate or vitamin E on the survival of mice exposed to phosgene. *J Appl Toxicol* 2001; **21(1)**:33-39
41. The Acute Respiratory Distress Syndrome Network. Ventilation with lower tidal volumes as compared with traditional tidal volumes for Acute Lung Injury and the Acute Respiratory Distress Syndrome. *New Eng J Med* 2000;**342(18)**: 1301-1308