1.1 BACKGROUND

Inhalation exposure to various toxic agents is a common phenomenon in most of the developing/developed nations leading to acute damage to the respiratory tract in large numbers of a population (Miller and Chang, 2003; Rabinowitz and Siegel, 2002). These toxic agents include toxic industrial chemicals (TICs), and other environmental pollutants. TICs are defined as the substances which are produced and used by industry for various purposes and that, because of its chemical, physical or biological properties, poses a potential risk for life, health, the environment, or property when not properly contained (Hincal and Erkekoglu, 2006). TICs also include a wide variety of lung-damaging chemical agents, which produce a toxic inhalational injury by attacking lung tissues upon inhalation. TICs are commonly found in communities of industrialized nations that manufacture chemicals, petroleum, textiles, plastics, fertilizers, paper, pesticides, vapors of volatile chemicals used in the work place. They are generally stored and transported as liquids and deployed as either liquid aerosols or vapors and victims are usually exposed to these agents via skin (liquid and high vapor concentrations), eyes (liquid or vapor), or respiratory tract (vapor inhalation) (Borrelli, 2007). These chemical agents may also pose a threat of being used as chemical agents for inflicting severe morbidity and mortality among civilian and defence personnel by way of intentional chemical. Their extensive industrial use easy availability and inexpensive nature make them an obvious choice for terrorists. Deliberate or unintentional release of TICs in sufficient quantities has great potential of hazard and even mass destruction capability on or off the battlefield (Tuorinsky and Shirley, 2008). This type of threat is specific for the 20th century and onwards, recognition is relatively new, and although it is generally underestimated, the terms “toxic warfare” or “chemical warfare without chemical weapons” are now frequently used to refer to the threat potential of TICs.

Besides TICs, other environmental pollutants which includes; cyanide, methyl isocynate (MIC), ammonia, chlorine, smoke inhalation, fire exhausts, radioactive radon gas, and vapors from paints, varnishes, petrochemicals, and many other airborne toxic products are also major threat to human health (Lane et al., 2006; Maynard et al., 1999; Sciuto et al., 2003; Smith et al., 2004). The United Nations Environment Programme (UNEP) has estimated that globally 1.1 billion people breathe unhealthy air (UNEP, 2002). Epidemiological studies have shown that
concentrations of ambient air particles are associated with a wide range of effects on human health, especially on the cardio-respiratory system (Dockery et al., 1996). A growing body of evidence has indicated that particulate pollution has increased daily deaths and hospital admissions throughout the world (Pope et al., 1995; Zanobetti et al., 2001). Beside respiratory diseases environmental pollutants adversely affect the cardiovascular system of the body (Brook et al., 2004; Pope et al., 2004).

Long-term exposure to these toxicants increases the risk of respiratory illnesses such as allergies, asthma, chronic obstructive pulmonary disease, lung cancer and interstitial lung diseases (acute lung injuries/ pulmonary edema) world-wide. Children and the elderly are particularly vulnerable to the health effects of these toxicants (www.niehs.nih.gov; Jamison, 2006). Whenever these toxicants are absorbed by inhalation they readily penetrate respiratory system leading to mucous membrane as well as immediate eye, nose, airway irritation causing acute damage to the respiratory tract (Greenwood, 2006; Rabinowitz and Siegel, 2002). The large surface area of lungs (100 m$^2$) coupled with the high respiration rate (15 l/min) provide significant opportunity for the entry of toxicants. Direct alveolar toxicity of these toxic agents increases capillary permeability causing pulmonary edema. Pulmonary edema follows a clinically latent period (24-48 hours) duration of exposure and depends primarily on the intensity of exposure (i.e., the concentration), but also partly on the physical activity of the exposed individual (White, 2002).

1.2 PULMONARY EDEMA
Pulmonary edema may be defined as swelling and/or fluid accumulation in the air spaces and parenchyma of the lungs caused by extravasation of fluid from the pulmonary vasculature (Fromm et al., 1995; Mattu et al., 2000). Pulmonary edema occurs when the alveoli fill up with excess fluid seeped out of the blood vessels in the lung instead of air causing problems with the exchange of gas (oxygen and carbon dioxide), resulting in breathing difficulty and poor oxygenation of blood i.e. hypoxia. Sometimes, this can be referred to as "water in the lungs" when describing the condition to patients. It leads to impaired gas exchange accompanied by severe respiratory distress, cold sweat, cyanosis, elevated blood pressure, palpitations and crackles over the lungs with oxygen saturation frequently less than 90% on room air prior to
treatment and may cause respiratory failure (Mattu et al., 2005; Swedberg et al., 2005). According to the World Health Organisation, generally 1-2% population is suffering from pulmonary edema worldwide, a life threatening disease, with a 12% in-hospital and 40% out-patient mortality (Roguin et al., 2000). Pulmonary edema is either cardiogenic, due to high pressure in the blood vessels of the lung leading to poor heart function and failure of the heart to remove fluid from lung circulation, or non-cardiogenic which is due to direct injury to lung parenchyma (Gonzales and Verin, 2012).

Pulmonary edema, especially in the acute setting, can lead to respiratory failure, cardiac arrest due to hypoxia, and death. Respiratory failure is a syndrome in which the respiratory system fails in one or both of its gas exchange functions i.e. oxygenation and carbon dioxide elimination and classified as either hypoxemic or hypercapnic (Grippi, 1998). The response to hypoxia is vasoconstriction causing difficulty in breathing which may lead to respiratory failure. Hypoxic pulmonary vasoconstriction is a paradoxical, physiological phenomenon in which pulmonary arteries constrict in the presence of hypoxia (low oxygen levels) without hypercapnia (high carbon dioxide levels), redirecting blood flow to alveoli with higher oxygen content (Moudgil et al., 2005; Sommer et al., 2008). Acute respiratory failure is characterized by life-threatening derangements in arterial blood gases and acid-base status, while chronic respiratory failure are less dramatic and may not be as readily apparent (Roussos and Koutsoukou, 2003).

1.2.1 Cardiogenic Pulmonary Edema

In cardiogenic pulmonary edema (CPE), a high pulmonary capillary pressure (as estimated clinically from pulmonary artery wedge pressure) is responsible for the abnormal fluid movement (Adair, 2001). Congestive heart failure due to poor heart pumping function (arising from various causes such as arrhythmias and diseases or weakness of the heart muscle), heart attacks, or abnormal heart valves can lead to accumulation of more than the usual amount of blood in the blood vessels of the lungs (National heart and lung disease, DCI, 2008). This can, in turn, causes the fluid from the blood vessels to be pushed out to the alveoli as the pressure builds up. In CPE, accumulation of fluid and protein in the alveolar space leads to decreased diffusing capacity, hypoxemia, and shortness of breath.
1.2.2 Non-cardiogenic Pulmonary Edema

Non-cardiogenic pulmonary edema (NPE) is also known as acute lung injuries (ALI) or acute respiratory distress syndrome (ARDS) or permeability pulmonary edema (PPE). This entity was first recognized and described by the military in relation to battlefield casualties in World War I and World War II (Perinea, 2003). NPE is a specific form of pulmonary edema that results from an increase in permeability of the normal alveolar–capillary barrier of the pulmonary capillary membrane due to direct or an indirect pathologic insult to lungs (Kilpatrick et al., 2011; Zimmerman and McIntyre, 2004). Alveolar capillary membrane becomes damaged and leaky that allows water and proteins to move freely from intravascular to the interstitial space, leading to accumulation of fluid and inflammatory blood cells in the lung (Goodman, 1996). Thus, the concentration of protein is almost identical in these two compartments. This situation is in contrast to that of the patient with cardiogenic pulmonary edema, in whom the protein concentration in the interstitial fluid is substantially less than within the intravascular space (Ware, 2010).

NPE is a condition that is associated with high morbidity and mortality which can be caused by different agents including inhalation injuries, smoke inhalation, inhalation of toxic gases, drowning, acute glomerulonephritis, fluid overload, allergic reaction, blood transfusion adverse reactions, heart bypass surgery adverse reaction, severe infection, pulmonary contusion, i.e., high-energy trauma, aspiration, certain types of medication, upper airway obstruction, i.e. negative pressure pulmonary edema, reperfusion injury, i.e. post pulmonary thromboendarterectomy or lung transplantation, adult respiratory distress syndrome and high altitude pulmonary edema in which factors other than elevated capillary pressure are responsible for protein and fluid accumulation in the alveoli (Bates, 2007; Luks, 2008).

1.2.3 Pathophysiological Mechanism

A complete understanding of the etiologies and mechanisms of development of NPE is necessary to help outline a proper therapeutic plan for patients that may develop this serious complication. NPE shows symptoms like extreme shortness of breath or difficulty in breathing, feeling of suffocation or drowning, wheezing or gasping for breath, anxiety, restlessness, a sense of apprehension, cough that produces frothy sputum that may be tinged with blood, excessive sweating and pale skin (Sartori et al., 2010). A classical sign of NPE is the production of pink
frothy sputum which leads to coma and even death, in general the main complication of hypoxia. The outstanding feature of acute lung injury caused by lung damaging agents is massive pulmonary edema. This is preceded by damage to the bronchiolar epithelium, development of patchy areas of emphysema, partial atelectasis, and edema of the perivascular connective tissue. The trachea and bronchi are usually normal in appearance. In gradually developing pulmonary edema, symptoms like nocturia (frequent urination at night), ankle edema, orthopnea and paroxysmal nocturnal dyspnea occur (Fig. 1.1). NPE is characterized by an extensive neutrophil influx in the lung, expression of pro-inflammatory mediators and damage of the lung epithelium and endothelium (Bdeir et al., 2010; Goodman et al., 1996). Reactive oxygen species (ROS) plays an important role in pulmonary vascular endothelial damage and is hypothesized to be responsible for clinical manifestation of NPE.

Fig. 1.1: Schematic diagram showing the mechanisms of pulmonary edema by inhaled toxicants
1.3 CURRENT THERAPY AND ITS LIMITATIONS

Non-cardiogenic pulmonary edema is a serious condition that usually requires hospitalization and intensive care. Although pulmonary edema can sometimes prove fatal, the outlook is often good when prompt treatment is received along with therapy for the underlying problem. Mortality rates for severe NPE have been reported to range from 50% to 70% in the past but are now declining with optimized treatment (Nuckton et al., 2002). Patients at increased risk include patients greater than 70 years old (Milberg et al., 1995), patients with associated dysfunction of other organ systems, patients with alcohol dependency, and patients with septic shock (Ely et al., 2001). Despite increases in knowledge about pathophysiological mechanisms that occur following edema, it has been a major challenge to evaluate NPE using non-invasive methods/techniques and develop effective therapeutic strategies for its medical management. Even though enormous efforts have focused on the development of drugs to limit NPE/lung damage caused by inhaled toxicants, there are currently no known measures to correct the permeability abnormality in NPE. Clinical management involves primarily supportive measures to maintain cellular and metabolic function while waiting for the acute lung injury to resolve (Fulkerson et al., 1996).

Oxygen therapy, positive pressure ventilation and bronchodilators are used as supportive care in NPE (Adhikari et al., 2007). There are only few pharmacologic agents which have been found to be effective in the treatment of NPE. Nitroglycerin and diuretics, such as furosemide (Lasix) which dilates the veins in lungs and elsewhere in body, are used as preload reducers, which decrease fluid pressure going into heart and lungs (Wheeler and Bernard, 2007). Nitroprusside (Nitropress), enalapril (Vasotec) and captopril (Capoten) are used as afterload reducers as they dilate the peripheral vessels and take a pressure load off the left ventricle. Corticosteroids, antioxidants, non-steroidal anti-inflammatory agents, prostaglandin E, antiendotoxin and anticytokine therapy, exogenous surfactant and pentoxifylline have been examined in clinical trials but so far do not confer a survival benefit (Anzueto et al., 1994; Bernard et al., 1997; Jepsen et al., 1992). Currently there are no clinically approved agents that can reduce pulmonary and airway cell dropout and avert the transition to pulmonary and/or airway fibrosis. In addition, there are few pressing lacunae in the presently used system of drugs viz; slow action, severe
systemic effect on high doses, and the fact that therapy does not address the primary issue of resolving the lung injury; the effort is directed towards clearance of edema only.

1.4 PULMONARY DRUG DELIVERY

Pulmonary drug delivery being a non-invasive delivery system is increasingly being used for effective local therapy and systemic administration of many therapeutic drugs for treating various disorders (Ali et al., 2009; Bhavna et al., 2009). The advantages of using pulmonary route for treatment includes; a) avoidance of first-pass metabolism (Carvalho et al., 2011; Patton and Byron, 2007), b) reduced dose since only a local effect is needed most of the times, and systemic distribution of the drug in therapeutic doses is not required, c) direct and fast action, d) high safety index i.e. reduction in side effects because of virtually no therapeutic drug concentration in blood, and e) increased patient compliance due to its non-invasive nature (Gill et al., 2007; Mansour et al., 2009). The large absorptive surface area of the lungs, extensive vasculature and thin alveolar epithelial barrier allow drugs to be delivered directly to tracheobronchial tree and alveoli of lungs making pulmonary route an alternative option for systemic drug delivery (Rodrigo, 2003). Apart from safety, the route offers the advantage that a number of drugs can be given simultaneously to provide a multi-directional approach. A very high concentration gradient can be built up in the lung spaces without the drug reaching toxic concentration in the blood. It has recently been reported that inhalation of vasodilators, including nebulized NO donors can selectively reduce various pulmonary inflammatory responses such as those seen in asthma, chronic obstructive pulmonary disorder (COPD) and pulmonary hypertension (PHT) (Xia et al., 2006).

Drug delivery to the lungs can be achieved by means of various devices such as nebulizers, metered dose inhalers and dry powder inhalers. Nebulizers are particularly gaining attention because of potential advantages such as stability, small droplets size, easy to formulate, low cost, better targeting efficiency, instant local action and higher pharmacological action in the lung at a significantly reduced dose, leading to reduction in adverse effects (Hess, 2008). However, routine inhalation through nebulization may not be useful because the drug gets deposited in pharynx and stomach, followed by tracheo-bronchial system, with alveoli receiving only 5-10%
of the inhaled dose. Thus 90% of delivered dose has no therapeutic value and contributes to side-effects only. The mean diameter of conventional drug aerosols is in the range of 5-10 microns which is not suited for alveolar drug deposition (Bhavna et al., 2009).

Pulmonary drug administration imposes stringent requirement on the delivery device; since the particle size of inhaled drug greatly influences its localization and thus the degree of its absorption from the lungs. Performance of an inhalable system mainly depends on the physical characteristics of particles such as mass median aerodynamic diameter (MMAD) which in turn depends upon shape, size and density of aerosolized particles (Chow et al., 2007; Khilnani and Banga, 2008). The proportion of aerosol particles in the respirable range (MMAD between 1 and 5 µm) is an important determinant of its deposition efficiency in the lower respiratory tract (Coates et al., 2007; Rogueda and Traini, 2007; Surez and Hickey, 2000; Telko and Hickey, 2005). Over the last 20 years the research focus within the field of respiratory drug delivery has broadened to include a wider range of potential applications for inhalation by delivering drugs not just onto the airway but across it (Gonda, 2000; Sanjar and Matthew, 2001).

Previous studies and data from our laboratory have shown that conventionally used micronized particles when inhaled, do not reach the lung spaces in adequate concentration. Micronized drugs have the tendency to remain in oropharyngeal region, which are later swallowed through mucociliary route into the gastrointestinal tract, resulting in almost 100% inactivation by liver (Grenha et al., 2005). Since efficiency of pulmonary drug delivery is a problem considering that only approximately 5-10% of the inhaled micronized drug powder reaches the alveoli (Newman and Wilding, 1998), it is necessary to increase peripheral lung deposition to achieve better therapeutics. This can be done by nano-sizing, or reducing the size of drug to submicron sized aerosol, thereby increasing the efficiency of treatment by attaining the required concentration (Ali et al., 2009; Bhavna et al., 2009; Chow, 2007; Kumar et al., 2011).
1.5 NON-INVASIVE APPROACHES IN DRUG DEVELOPMENT

The need: current techniques to evaluate the efficacy of potential treatments for respiratory diseases in small animal models are generally invasive and terminal. To study specific aspects of human respiratory diseases and its treatment it is necessary to measure the induced symptoms and the impairment of lung function in living animals. The inflammatory status of the lung is routinely inferred from post mortem analyses of broncho-alveolar lavage (BAL) fluid. Occasionally, time consuming histological analysis is also performed.

1.5.1 Gamma Scintigraphy

Diagnostic imaging using gamma scintigraphy is a well-established procedure in nuclear medicine and has been used extensively in development of inhalation drug delivery systems for over twenty years (Lim et al., 2007). The non-invasive imaging technique of gamma scintigraphy was developed originally for use in diagnostic tests in nuclear medicine (David, 2005). Currently it is the only non-invasive method capable of providing human data on total and regional lung deposition and mucociliary clearance. There are likely to be increasing demands for such studies in this area of rapid scientific advancement. It is the method of choice for investigating the fate of pharmacological dosage form in humans and demonstrating how a delivery system is behaving inside the system and whether it is behaving as intended or not (Brooks et al., 2005; Youngho, 2008). Specific radiopharmaceuticals which localize in different organs and which are visualized by gamma camera are used to provide vital information about the structure and function of various body systems. In contrast to radiological imaging that is based on transmission of radiation, gamma scintigraphy uses emitted radiation.

1.5.2 Magnetic Resonance Imaging

Magnetic resonance imaging (MRI), or nuclear magnetic resonance imaging (NMRI), or magnetic resonance tomography (MRT) is a medical imaging technique used in radiology to visualize detailed internal structures. The good contrast it provides between the different soft tissues of the body makes it especially useful in imaging of lung, brain, muscles, heart, and cancer as compared to other medical imaging techniques such as computed tomography (CT) or X-rays. Unlike CT scans or traditional X-rays, MRI uses no ionizing radiation. Instead it uses a powerful magnetic field to align the magnetization of some atoms in the body, and then uses
radio frequency fields to systematically alter the alignment of this magnetization. This causes the nuclei to produce a rotating magnetic field detectable by the scanner and this information is recorded to construct an image of the scanned area of the body. Animal MRI is a relative new technology that assesses the effect of inflammation, mucus secretion, airway and vascular remodeling (Beckmann et al., 2001; Tigani et al., 2007), and parenchymal destruction (Karmouty et al., 2006) in the rat lung serving as important tool to study a variety of respiratory diseases. Another advantage is that the disease progression is followed in the same animal. The observed MRI fluid signal generally correlates with perivascular edema assessed by histology (Tigani et al., 2003). Flexibility of MRI can therefore be explored to obtain anatomical and functional information of the rat lung, with the scope of developing a non-invasive means of analysis.

1.6 AIMS AND OBJECTIVES
Possibility of chemical accidents involving toxic industrial chemical, environmental pollutant as well chemical warfare agents always exist where-ever these chemicals are used. During exposure, 60-70% of toxic chemicals is inhaled either as aerosols or colloidal particles and absorbed therein causing non-cardiogenic pulmonary edema/ acute lung injury. As mentioned above the treatments available are not very effective and are slow acting. Since during exposure, 60-70% toxicants directly reach the lungs, a delivery system is required that can trap or neutralize toxic inhaled substances at entry point only i.e. in the lungs directly.

Institute of Nuclear Medicine and Allied Sciences (INMAS) is working in different areas of drug development related to high altitude problems, low intensity conflicts and medical management of nuclear biological and chemical disasters. Several formulations have been developed and evaluated under these projects for addressing various medical medical problems faced by Armed Forces Personnel as well as general population. These include oral tablets, injectables as well as inhalation based formulations. Nano/ submicron-based inhalation formulations viz; Salbutamol Sulphate, Calcium disodium-EDTA, Alpha Keto Glutaric Acid, and Sodium Alendronate, including nebulizable respiratory solutions and nano-dry powder inhaler (DPI) have been developed which have shown to deposit in lungs in higher concentration, thereby showing their distinct clinical advantage over the conventional micronized formulations (Ali et al., 2012;
Faiyazuddin et al., 2012; Kumar et al., 2011; Sultana et al., 2011, 2012). Efficacy of many of these inhalable formulations in pulmonary vasodilatation has been studied. However their role in NPE remains undetermined.

The goal of present study was to explore methods to evaluate non-cardiogenic pulmonary edema/acute lung injuries and to reduce those using specific and non-specific therapies. Specific therapy includes, role of Sub-micronic Alpha Ketoglutaric Acid respiratory formulation against ammonia induced acute lung injuries and oxidative damage while in non-specific therapy, the prophylactic/therapeutic potential of sub-micronic Sodium Nitrite and Fluticasone propionate respiratory formulation against passive cigarette smoke induced oxidative stress and lung injuries will be evaluated.

It may be noted that physicochemical properties and toxicological profile of a sub-micronic particle differs considerably as a result of its smaller size and unique form as compared to larger micronized particles composed of same material (Borm et al., 2006; Nel et al., 2006). Therefore systematic pre-clinical studies have to be carried out post sub-micron sizing of the formulations before it can be evaluated in clinical setting. Pre-clinical safety and efficacy studies are therefore required in suitable animal models to get necessary approval from drug regulatory authority.

The present study has therefore been done with the following objectives:

- To create animal model of non-cardiogenic pulmonary edema.
- Invasive and non-invasive methods for evaluation for pulmonary edema.
- To evaluate efficacy and role of drugs against non-cardiogenic pulmonary edema.