SUMMARY

Non-cardiogenic pulmonary edema/ acute lung injuries are one of the leading cause of morbidity and mortality and major cause of long term disability worldwide in industrial and chemical accidents. Acute lung injury may be induced by pneumonia, sepsis, multiple trauma, aspiration of gastric contents and severe burns. It is a critical illness syndrome marked by excessive production of pro-inflammatory mediators, massive infiltration of leukocytes, and rapid alveolar injury, resulting in respiratory failure and high short-term mortality. It has a substantial impact on public health with a very high hospital mortality rate of 38% to 50% and associated morbidity, and it is estimated that the annual incidence of acute lung injuries will double in the next 25 years, as the population ages. Environmental, industrial, as well warfare toxicants viz; ammonia, chlorine, hydrogen cyanide, phosgene, passive cigarette smoke, petrochemicals, methyl isocyanate, sulfur mustard, and nitrogen mustard can be encountered accidentally or intentionally during industrial processes and transportation which can adversely affect human health. These chemicals may prove lethal if exposed to high concentration or for a longer duration in low concentrations. Toxicants inhalation is well known for their deleterious effects on lungs resulting in various respiratory disorders including pulmonary emphysema, chronic obstructive pulmonary disease, leading to non-cardiogenic pulmonary edema.

The pulmonary vasculature is a major target of oxidative stress and is susceptible to oxidative damage, playing a critical role in the pathogenesis of acute lung injuries following inhalation of toxicants. Oxidative stress plays a fundamental role in acute lung injury because of large consumption of oxygen by the lungs. Oxidative stress culminates due to an imbalance between pro-oxidants and antioxidants and consequent excessive production of reactive oxygen species. Reactive oxygen species are implicated in a number of disease processes, whereby they mediate damage to cell structures, including lipids, membranes, proteins, and DNA. Therefore, it is believed that pharmacological modification of oxidative damage is one of the most promising avenues for inhalation therapy. Pulmonary damage protection might be provided by agents that interfere with factors involved in pathogenesis.

Despite recent considerable attention and advances in antibiotic therapy and intensive care, the prognosis of acute lung injuries remains poor to the approach of protection of lung injuries. To date, most basic and clinical trials have focused on antioxidants and anti-inflammatory agents. Currently, there is no specific pharmacologic approach for mitigating the effects of these toxic
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chemicals inhalation on the lung. Since a toxicological mass event is usually unpredictable, adequate preparedness is of utmost importance. The development of an effective therapy, therefore, has important implication for the planning of critical care services, rehabilitation, and resource provision. Nitric oxide donors, steroids and toxicants neutralizing agents in the form of nano/ submicron sized therapeutic agents can be used as a newer dimension in the management of pulmonary injuries. There are many animal models available to investigate mechanisms of lung injuries as well new preventive or therapeutic strategies. The use of appropriate animal models is essential for understanding of deleterious mechanisms involved in a particular disease, and to identify the potential efficiency of therapeutic strategies against it.

The pulmonary route of administration for nano/ submicron sized therapeutic agents has been used for many years for systemic as well as local action of lung diseases. About 60-70% of toxic substances as aerosol or colloidal particles reach directly to the lung. Inhalation based targeting of a therapeutic agents will trap them at entry point without making them available for systemic circulation where they can stay for prolonged period and cause tissue damage. Many of nano/ submicron sized therapeutic agents have shown prophylactic/ therapeutic potential against pulmonary damage in a variety of bioassay systems and animal models. The lung deposition characteristics and efficacy of therapeutic agents in aerosol form depends largely on the particle or droplet size. Generally, the smaller the particle, greater is its chance of peripheral penetration and retention.

The present work concerns with induction and evaluation of non-cardiogenic pulmonary edema/ acute lung injuries caused by inhaled toxic chemicals (ammonia, passive cigarette smoke, and kerosene fumes) in suitable small animal models (rats/ rabbits) using invasive as well non-invasive techniques. Further, toxicological and pharmacological evaluation of developed sub-micronized particles based respiratory formulations and their prophylactic and therapeutic role has also been evaluated in small animal models. In our studies, the respiratory fluid composition for nebulization of the developed sub-micronic formulations was optimized on the basis of particle size of aerosolized droplet using particle size analyzer. The respiratory fraction of developed sub-micronic respiratory formulations was performed by ACI using gamma scintigraphy and UV spectroscopy and compared with conventionally available micronic formulations.
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Chapter I, introduces the subject, discusses the scope of work in relation to the existing knowledge, highlighting the innovations and techniques involved, and presents the objectives of the work envisaged in accordance to the synopsis submitted.

Chapter II, presents an in depth review of literature covering the following areas: pulmonary edema, non-cardiogenic pulmonary edema/ acute lung injuries, its current therapies, its mechanism of pathophysiology, current therapies used for its treatment and their limitations as well drug profile of therapeutic agents used in the present work.

Chapter III, contains materials and methods dedicated to experimental design for the experiments performed under the study. Different toxic chemical agents have been used to induce non-cardiogenic pulmonary edema in small animal models, evaluation using invasive and non-invasive techniques and toxicological and pharmacological evaluation of developed sub-micronic respiratory formulations of therapeutic agents.

Chapter IV-VII, embody the actual experimental design, material and methods and work done, exploring the toxicological and pharmacological evaluation of different developed sub-micronic respiratory formulations and their role against non-cardiogenic pulmonary edema/ acute lung injuries. It includes a series of independent experiments that work upon a single new application of induction of acute lung injuries/ oxidative stress and therefore contain a separate Introduction, Treatment regimen, Results and Discussion subsections. Since the number of such independent sections is indeed very high, the descriptions in the subsection have been kept concise.

Chapter-IV, we have investigated, the particle size of developed sub-micronic respiratory formulation of AKG using particle size analyzer, its in vitro and in vivo lung deposition and retention using Anderson Cascade Impactor and human gamma scintigraphy respectively. Thereafter in vivo toxicological and pharmacological evaluation of developed and optimized formulation against ammonia induced non-cardiogenic pulmonary edema/ acute lung injuries in male Sprague Dawley rats were carried out. Sub-acute inhalation toxicity studies conducted revealed no changes in any morphological, hematological and biochemical parameters when compared with control. General health, food and water consumption remained similar in the three test groups of AKG post-treatment. No death was observed in any group of post-inhalation treatment. No adverse effects were observed on animal body weights gain in AKG inhalation groups of animals as compared to control. Lung toxicity biomarkers parameters analyzed in BAL fluid showed no significant alteration and these were similar to the control. Organ/ body weight
ratio and histopathological findings in vital organs (heart, kidney, liver, lung and spleen) showed that there was no significant evidence of damage to the lung alveoli and parenchyma in sub-micronic AKG respiratory formulation treated groups of animals confirming its safety.

In this study, we have also investigated the therapeutic effects of sub-micronic AKG respiratory formulation on functional outcome, lung damage and pulmonary vascularity in the edematous lungs caused by ammonia inhalation. Animals were exposed with liquid ammonia aerosols (3000 ppm) for 15 min and then post-treatment of sub-micronic AKG respiratory formulation inhalation was provided. After ammonia inhalation and post-treatment of AKG, gross pathology, body weight variation, hematological, serum biochemistry analysis in blood samples, lung wet/dry weight ratio analysis and activities of antioxidant enzymes were measured in BAL fluid. Histopathology analysis of lung tissues was also performed. The inhalation of ammonia showed marked reduction in body weight gain. Significant changes in morphological, hematological and biochemical parameters were observed too. The effects were however reduced in AKG treated group. A significantly depleted activity of antioxidant enzymes and content of glutathione in ammonia inhalation group were protected significantly in ammonia inhalation group post-treated with sub-micronic AKG respiratory formulation. Conversely, the elevated levels of total cell counts, total protein, LDH, SOD, and LPO in ammonia inhalation group were attenuated significantly with AKG treatment. The results indicated that sub-micronic AKG inhalation treatment protected the lung from damage caused by ammonia inhalation.

**Chapter-V**, deals with particle size analysis, its *in vitro* and *in vivo* lung deposition and retention of developed sub-micronic Sodium Nitrite respiratory formulation. Subacute inhalation toxicity and prophylactic efficacy against passive cigarette smoke induced oxidative stress and lung injuries were carried out. After following treatment protocol for toxicity evaluation animals were observed for morphological and behavioral parameters and then sacrificed for analysis of hematological and serum biochemistry analysis. BAL fluid was collected to observe lung toxicity biomarkers. Organ/ body weight ratio and histopathological evaluation of vital organs (Heart, Kidney, Liver, Lung, and Spleen) was performed to observe drug related adverse effects. Toxicity studies conducted according to Schedule-‘Y’, revealed no changes in any morphological, hematological and biochemical parameters. BAL fluid analysis as well as histological parameters showed no significant changes in sub-micronic Sodium Nitrite formulation inhaled group when compared with control, thereby confirming its safety.
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Prophylactic effect of sub-micronic Sodium Nitrite respiratory formulation against passive cigarette smoke induced oxidative stress and lung injuries was also evaluated. We presented evidences documenting that inhalation of sub-micronic Sodium Nitrite respiratory formulation mitigates smoke effects, suggesting its efficacy to reduce lung injuries following oxidative stress. The study highlights the prophylactic potential and point out to its importance as a new therapeutic agent in developing a novel prophylactic/therapeutic option for smoke exposed victims as well as members of the rescue team, including fire-fighters.

Chapter-VI, in the similar ways explain the particle size analysis, in vitro and in vivo lung deposition of developed sub-micronic respiratory formulations of Fluticasone propionate its toxicological and pharmacological evaluation against passive cigarette smoke induced oxidative stress and lung injuries in experimental animals. During subacute inhalation toxicity studies no abnormal signs or symptoms were recorded in any of the animals. General health, food, water consumption, body weight gain remained similar in the three test groups of respiratory formulations of sub-micronic Fluticasone propionate as compared to control. Hematological and serum biochemistry parameters results were within the normal range in treated animals and there were no significant changes as compared to control. Lung toxicity biomarkers analyzed in BAL fluid were similar to the control showing that no edema/injuries were induced by drug inhalation. Organ/body weight ratio and histological observations of vital organs also showed no adverse effects of sub-micronic Fluticasone propionate inhalation treatment confirming its safety. Pharmacological evaluation showed that inhalation of sub-micronic Fluticasone propionate respiratory formulation ameliorated oxidative stress and lung injuries caused by smoke inhalation by decreasing inflammatory action.

Chapter-VII, The aim of this study was induction and evaluation of non-cardiogenic pulmonary edema/cardio-pulmonary stress through kerosene fume inhalation in rabbits using invasive and non-invasive techniques. This chapter also deals with the study of effect of petrochemicals in long term occupational exposure to petrol filling station workers using pulse oxymetry as a non-invasive cardio-pulmonary health analysis tool. The results showed significant changes in pulse rate and blood oxygen saturation levels (SpO₂) of long term exposure in petrol filling station workers due to petrochemicals effects on lungs. These animal models of acute lung injuries can be used to understand the mechanism and pathogenesis of petrochemicals to develop prophylactic/therapeutic strategies for its treatment management.
Current project work was especially designed to emphasize to contribution to illustrate the usefulness of gamma scintigraphy, magnetic resonance imaging (MRI) and pulse oximeter to obtain anatomical and functional information of the lung, with the scope of developing a non-invasive approach for the routine testing of drugs in small animal models (rat/ rabbits) of pulmonary diseases. In our studies, $^{99m}$Tc-DTPA clearance was increased in ammonia and smoke induced non-cardiogenic pulmonary edema/ acute lung injuries as shown by gamma scintigraphy. To the best of our knowledge, there are no published data about the effects of ammonia, passive cigarette smoke on alveolar clearance determined by gamma scintigraphy after intratracheally instilling radionuclides ($^{99m}$Tc-DTPA) in lungs. The effect of these toxicants on $^{99m}$Tc-DTPA clearance was considered due to its impairment effect on alveolar permeability. In our studies, prophylactic/ therapeutic potential of different developed sub-micronic respiratory formulations against acute lung injuries induced by chemical toxicants have been evaluated using this technique.

We have also used animal MRI and pulse oxymetry as a non-invasive tool for evaluation of acute lung injuries caused due to ammonia and smoke inhalation which can be further used in evaluation of various therapeutic agents. These non-invasive approaches can be used to decrease the number of animals required for each experiment. The main asset of these techniques is their ability to detect variety of changes in lungs in non-invasive manner under the conditions of spontaneous breathing. This characteristic of these techniques provide a clear advantage in pulmonary drug delivery and research as the same animal can be used as its own control for baseline measurements and can be monitored repeatedly over time without changing its physiology, the only restriction being its exposure to the anesthetics.
CONCLUSIONS

The following conclusions can be drawn from the results obtained from the present project work:

- Non-cardiogenic pulmonary edema/ acute lung injuries were induced in small animal models (rats/ rabbits) using inhaled chemical toxicants (ammonia) and environmental pollutants (passive cigarette smoke and petrochemicals) which were evaluated using invasive as well as non-invasive techniques (Gamma Scintigraphy, Animal MRI and Pulse Oxymeter).
- Sub-micronic respiratory formulations of Alpha Ketoglutaric Acid, Sodium Nitrite, and Fluticasone Propionate were prepared in ethanol and saline for inhalation.
- In vitro lung deposition studies by cascade impactor exhibited significant lung deposition behavior when compared with micronized drugs respiratory formulations. Respirable fraction and MMAD of designed formulations was found suitable for deep alveolar targeting.
- Sub-acute inhalation toxicity studies of developed sub-micronic respiratory formulations of these therapeutic agents were carried out according to Schedule-‘Y’ guidelines which revealed no significant changes in animal’s macro and micro, hematological, biochemical and histological studies of vital organs of treated animals confirming their safety.
- Therapeutic potential of sub-micronic Alpha Ketoglutaric Acid respiratory formulation in amelioration of ammonia induced acute lung injuries was assessed using invasive as well non-invasive techniques.
- Prophylactic potential of sub-micronic Sodium Nitrite and therapeutic efficacy of sub-micronic Fluticasone Propionate respiratory formulations against smoke induced oxidative stress and lung injuries was also carried out which showed promising results and findings.
- Gamma Scintigraphy using $^{99m}$Tc-DTPA, along with suitable biochemical markers have indicated prophylactic/ therapeutic efficacy of developed respiratory formulations against lung toxicants induced oxidative stress and acute lung injuries.
- Due to its low cost and short duration gamma scintigraphy may be more useful in monitoring new therapeutic interventions in lung injuries which can be used to minimize the number of animals sacrificed during drug development process.
ACHIEVEMENTS

The work done under this thesis conclusively proved the therapeutic role of three sub-micronic respiratory formulations (Alpha Ketoglutaric Acid, Sodium Nitrite, and Fluticasone propionate) against non-cardiogenic pulmonary edema/acute lung injuries induced by ammonia and passive cigarette smoke inhalation. Consequent to the present work done the organization (INMAS) was able to conduct preliminary human studies and get permission from Drug Controller of Government of India (DCGI) as a new drug for disease management. The data of this thesis work was used by institute to convince army authorities to use these drugs in field conditions. There is presently no known treatment for ammonia toxicity. In the course of this study a simulation model for ammonia inhalation toxicity and treatment is reported for the first time. Several new technologies viz: gamma scintigraphy, DTPA clearance and animal MRI have been introduced probably for the first time for animal toxicity studies pertaining to toxic pulmonary edema. The fact that gamma scintigraphy can accurately quantify the amount of inhaled drug in living animals will go a long way to validate such studies in future.