A acute lung injury (ALI) and its more severe form, the acute respiratory distress syndrome (ARDS), is defined as a ‘syndrome of acute pulmonary inflammation and increased capillary endothelial permeability’ and can occur in response to both direct (aspiration, pneumonia, toxic inhalation) and systemic (sepsis, shock, cardiopulmonary bypass) challenge. ALI is associated with a neutrophil-mediated inflammatory response that causes alveolar epithelium/endothelium breakdown, increased vascular permeability and edema. Among the different pulmonary changes during lung injury, the inactivation of surfactant or the imbalance of surfactant function are known to be of significant importance in the setting of ARDS. One of the most promising approaches to improve the surfactant function during ARDS is the application of exogenous surfactant via the airways. Indeed, the application of exogenous surfactant preparations in the treatment of severe ARDS resulted in an improved pulmonary oxygenation. However, it failed to reduce the mortality in large controlled clinical trials of ARDS so far. The reasons for the limited efficacy of surfactant replacement strategies that have thus far been employed in ALI and ARDS are not well understood. A better understanding of this process will be needed to devise more effective approaches to the restoration of surfactant function in critically ill adults. Therefore, the present work was envisaged to study the interplaying role of the surfactant in combating the LPS induced ARDS in a rat model.

A credible ARDS model was established through different LPS dose standardization viz. 50, 150, 450μg/animal given at various time intervals 18, 24, 48, 72, 96h. As evident from the results, the dose of 150μg/rat for a period of 72h was found adequate to generate the ARDS model as seen by the histological and various biochemical analyses.

After establishing the appropriate ARDS model, we checked the efficacy of two different types of exogenous surfactant (porcine derived natural surfactant and chemically prepared synthetic surfactant) in ARDS. For which, first of all the surfactant from pig BALF was isolated by density gradient method and the isolated fraction (surfactant) (P-SF) was biochemically analyzed. Three different sets of animal groups comprising of Control, LPS, LPS+P-SF and LPS+S-SF were carried out and the duration for both types of surfactant treatments was standardized viz. 2, 12, 24h. Out of the three time periods, 2h time period was selected for
surfactant treatment as maximum beneficial/protective effects were observed for both the surfactants, as clearly evident after histopathology and biochemical analyses.

H/E staining of lung tissue 72 h post LPS administration indicated inflammatory changes such as thickening of the alveolar septa and infiltration of inflammatory cells into the lung interstitium and alveolar spaces when compared with the buffer control. Surfactant treatment greatly diminished the histological signs of LPS-induced inflammation. The inflammatory cell population including neutrophil and macrophages were counted in BALF of control as well as various treated groups. These cells were found to be elevated in LPS treated group which was corrected by the surfactant treatments. The results indicate that these inflammatory cell populations play an important role in the ARDS development. MPO activity as a measure of neutrophil influx was also found to be in agreement with the above results. W/D lung weight ratio and inflammatory cytokines were noted maximum in the LPS only group while these values were minimally observed in the LPS + P-SF and LPS + S-SF group. LPS group showed a high RONS occurrence, however, with surfactant co-administration the production of RONS was found to be markedly reduced.

These observations therefore, clearly establish the LPS induced ARDS model as well as the preventive effects of exogenous surfactants in rats validated. For studying in detail the role of inflammatory proteins, and apoptotic proteins along with other signaling molecules in ARDS and evaluating the protective effects of surfactant during LPS induced ARDS, the present experiments were designed.

Relationships between lung function, and surfactant function and composition were examined during the evolution of acute lung injury. The levels of SP-A and SP-B in the lung tissue of animals in the various treatment groups were assessed by Western blotting and by immunolocalization. Changes were seen in the relative level of SP-A and SP-B after all the treatments. The expression of both the surfactant proteins were decreased with LPS treatment and reverted back to the control level by exogenous surfactant treatments.

We have seen the expression and localization of TNF-α, IL-1β, IL-2, IL-4, IFN-γ, MCP-1 and MIP-1β in the tissue of rats treated with LPS and surfactant groups by Western blot and immunofluorescence. Further, we have seen the gene expression for TNF-α, IL-1β, MCP-1 and MIP-1β by RT-PCR and also quantitate the levels of the cytokines in the BALF by ELISA. We
found that the expression of all the proinflammatory cytokines were higher with LPS treatment and subsequently suppressed by surfactant treatment.

Further, we studied the role of inflammation in ARDS. Various landmark molecular events leading to the inflammation were analyzed by Western immunoblot and immunohistochemical localization of inflammation activators such as COX-1, COX-2, iNOS, NF-κB, IKKα and IκB was also analyzed. In the present investigation, COX-2 activity as well as its expression displayed a pronounced change within 72 hours of endotoxin exposure and subsequently corrected with surfactant administration. The present study demonstrates that in the pathogenesis of ARDS, treatment with surfactant significantly (including both animal derived and synthetic surfactant) attenuates endotoxin-induced low environmental pH which generates NO released by iNOS. We also demonstrated that surfactant suppressed NF-κB transcriptional activation and translocation to the nucleus and significantly inhibited IκB phosphorylation and degradation by blocking IKKα kinase activity in the lung tissue. Therefore, limited acidification of lung tissue environment is sufficient to induce the expression of iNOS gene and the synthesis of NO. This effect is due to increased translocation of NF-kB to the nucleus and correlates well with the lung gene expression of iNOS and COX-2.

PI3-kinase and Akt play major role in cellular functions regulating the host defense and immune response. Our results showed modulation of PI3-kinase /Akt pathway after LPS treatment, which may be responsible for the observed inflammatory effects of LPS as PI3-kinase and Akt have been shown to participate in signaling pathways that lead to NF-κB activation and increased NF-κB-dependent transcription. The elevated levels of Akt as a result of PI3-kinase activation also seem responsible for the reduced levels of GSK-3β in the present study. The immunohistochemistry of Akt in paraffin sections from LPS group supported a high expression of Akt in both epithelial cells and alveolar macrophages, whereas only a few cells were seen expressing Akt in all the other groups. GSK-3β was reduced significantly by LPS treatment, however surfactant treatment improved the levels. Results from immunohistochemistry of GSK-3β coincided well with the results from protein expression study by Western blot. Therefore, PI-3 kinase and their downstream targets may provide novel therapeutic targets for intervention in the ARDS patients.

The mechanisms responsible for increased apoptosis in ALI/ARDS are poorly understood, although roles for Fas/FasL (extrinsic apoptosis), stress (intrinsic apoptosis) and
Summary and Conclusion

nitric oxide (NO) have been proposed. Various rate limiting molecular events leading to the apoptosis were analyzed which included role of reactive oxygen species (ROS), calcium homeostasis and mitochondrial dysfunction in terms of decreased membrane potential as well as mitochondrial permeability transition. In this study, we detected apoptotic hallmarks, such as activation of pro apoptotic signals like Apaf-1, cytochrome-c release, activation of caspases along with analyzing the status of cytoplasmic proteins like Bax and Bcl-2, PARP cleavage, Annexin V binding, DNA fragmentation, TUNEL and LDH release. Mitochondria are primary sources of ROS inside the cells. To verify that the effect of LPS on membrane potential paralleled the alterations in redox state in the cells, we examined ROS production using a fluorescent dye (DCFH-DA) and found that both the surfactants had inhibited the LPS stimulated ROS generation. Experiments in this study also substantiated that LPS induced apoptosis is followed by an elevated intracellular calcium level ([Ca^{2+}]) along with the mitochondrial membrane depolarization. These phenomena were found to promote the anti-apoptotic activity of Bcl-2 which was higher after surfactant treatment along with LPS and decreased translocation of Bax to the mitochondria. Our findings of marked expression of Bax suggest that LPS-associated apoptosis in ARDS may be facilitated by the induction of Bax. High levels of Bcl-2 prevent mitochondrial dysfunction and protect against apoptosis, whereas high levels of Bax promote apoptosis. Surfactant treatment on the other hand, proved beneficial in reversing the apoptotic promoting effects of LPS by initiating the anti-apoptotic signals as evident by the decreased expression of mitochondrial proteins (cytochrome c, Apaf-1) and decreased cleavage of PARP protein. Moreover, caspase 9 and 3 activities were also found to be lower in the surfactant treated groups further confirming the deactivation of apoptotic pathways. Additionally, morphological assessment and quantification of apoptosis were also done using fluorescent labeling of the type-II cells as well as alveolar macrophages along with the TUNEL assay. These results established the occurrence of more number of apoptotic cells in LPS treated group while in LPS+SF group, the incidence of apoptosis decreased with minimum presence of apoptotic bodies, as also seen by negligible DNA fragmentation (ladder formation) in the surfactant treated group.

We have explored the potential role for VEGF and PPAR-γ in the development of ARDS. We detected lower levels of VEGF in the lungs of animals with LPS dose compared with those from normal, or surfactant treated groups. The correlation between the severity of lung injury and VEGF levels suggests that changes in VEGF levels may reflect the degree of alveolar...
epithelial damage. In addition, with surfactant treatment the VEGF levels were reverted back to the control level. This down regulation of VEGF in the alveolar space may reflect the recovery from acute lung injury aimed at limiting endothelial permeability, and may participate in the decrease in capillary number that is observed during acute respiratory distress syndrome with potentially significant clinical consequences. To elucidate the functional role of PPAR-γ, the ligand dependent transcription factor, we next examined the effect of LPS on the induction of PPAR-γ expression in the rat lung tissues. We found that P-SF and S-SF markedly decreased the LPS-induced PPAR-γ production. Although the precise mechanisms by which PPAR-γ exerts anti-inflammatory effects are poorly understood, surfactant treatment conferred protection in rat model dependent on PPAR-γ and induction of VEGF production.

Therefore, it seems rational to administer exogenous surfactant to ARDS patients. The findings of this study have several potential implications for surfactant replacement strategies. It may be noted here that among the two surfactants tested, porcine surfactant was found to be most effective in preventing the LPS induced ARDS effects as studied for all the present experiments. Adequate surfactant intubation will require that surfactant is containing adequate amounts of both phospholipids and apoprotein components and delivered to the most affected lung segments in sufficiently high concentrations to overcome the inhibitory effects of plasma proteins (proteases) and other unidentified factors. During ARDS there may be ongoing degradation of surfactant apoproteins in the areas of most intense inflammation, supranormal quantities of apoproteins may need to be included in the surfactant replacement mixture to act as a reserve and to supplement existing surfactant in those alveoli. On the basis of the results observed in this animal model, one would predict that a non apoprotein-containing surfactant delivered by aerosolization would have limited efficacy in ARDS. On the other hand, apoprotein containing surfactant delivered by instillation in supraphysiological quantities might well have a positive impact in ARDS. However, this therapeutic approach is hampered by the costs; natural surfactants are being very expensive. For this reason more synthetic surfactants need to be developed. These synthetic products may include formulations that have significant anti-inflammatory effects, and are promising in animal models of acute lung injury as in ARDS. Till date, natural surfactants are however more effective than synthetic ones.

To conclude, the establishment of a small-animal model of ARDS and surfactant dysfunction secondary to LPS administration, coupled with a detailed multipronged
examination of the underlying processes, make a contribution to our understanding of ARDS and surfactant administration may represent a promising future therapeutic option for the reduction of ARDS. Whether these protective effects of surfactant application observed in animal model of lung injury can be extrapolated to the treatment of patients is the subject of various ongoing clinical trials.

Figure 5: Shows the overall effect of LPS and surfactant on the various signaling pathways which have been studied in the present study. Blue arrows show the effect of LPS treatment whereas green arrows show the effect of surfactant treatment. Activation is represented by triangular arrow-heads whereas repression is shown by flat arrow heads.