SUMMARY and CONCLUSIONS
Mineral fibers/particles induced lung damages are among the most widespread and prevalent occupational lung diseases in the world. Most likely as a consequence of the specific toxicokinetic properties of mineral fibers/particles such as, durability, specific fiber dimensions, chemical composition, surface properties and charge, disease may even progress after exposure has ended. Despite the world-wide diversity of mineral-rich areas and in the structure, physicochemical properties and related application of these minerals, they all share the probability to become airborne and eventually be inhaled.

Deposition of inhaled mineral fibers/particles in the lung is primarily dependent on size, shape and particle density, as well as on individual lung morphology and physiology. Generally, large particles (> 10 μm) will be filtered out of the inhaled airstream by the aerodynamic filters of the respiratory tract, whereas smaller particles are deposited in the tracheobronchial tree or alveoli. In the upper airways, dust particles are cleared by the mechanism of sneeze and cough, or by the mucociliary escalator and ultimately be swallowed. In the alveolar region, the majority of the
particles are ingested by phagocytotic cells such as alveolar macrophages (AM). If the length of the particle is too large, "frustrated phagocytosis" may occur characterized by an incomplete phagocytosis. This has been shown to result in an activation of the AM and during its activation a wide range of products including reactive oxygen species (ROS), cytokines, growth factors etc. are released. A state of "particle overload" may occur if more particles enter the lung than are cleared from it due to impairment of AM clearance. Ultrafine particles are considered to cross the epithelial barrier into the interstitium more readily and noteworthy, a relative low weight-dose of ultrafine particles may comprise a tremendous numerical dose.

Occupational exposure to mineral fibers can cause pulmonary fibrosis (known as asbestosis), benign pleural changes, lung cancer and malignant mesothelioma. Inspite of extensive research in this direction, the mechanism(s) by which mineral fibers cause these diseases is/are not fully understood. However, several studies have suggested that ROS play an essential mediating role in the pathogenesis of these diseases. Mineral fibers can induce ROS formation by atleast two different processes, in which (i) mineral fibers may augment ROS release by inflammatory cells and (ii) ferrous iron in fibers in the presence of hydrogen peroxide (H₂O₂) generates hydroxyl radicals (OH) via the Fenton reaction.

The protection of cells against damage by ROS is accomplished through both enzymatic and non-enzymatic means. Glutathione (GSH), a tripeptide and the most prevalent nonprotein intracellular thiol present in high concentrations in almost all living cells, protects the cells against oxidative damage. GSH redox system enzymes provide a formidable protective shield against oxidative damage. GSH detoxify a variety of electrophilic exogenous toxicants and their reactive intermediates formed intracellularly either spontaneously or enzymatically.
In the studies presented in this dissertation an attempt has been made to evaluate the cytotoxic, fibrogenic and carcinogenic potential of mineral fibers/particles in AM (phagocytotic cells), red blood cells (RBC) and lung tissue of exposed rats. The study was divided into five parts and is described in brief under the following heading.

1. **Cytotoxicity, prooxidant effects and antioxidant depletion in rat lung alveolar macrophages exposed to ultrafine titanium dioxide**

In order to understand the pulmonary toxicity of mineral particle ultrafine titanium dioxide (UF-TiO$_2$) various biochemical and chemical parameters were assayed. Single intratracheal exposure of UF-TiO$_2$ (2mg/rat) after 1, 4, 8 and 16 days caused a significant increase in AM population. The maximum increase in the number of AM population was recorded on day 8th of exposure which occurred due to recruitment of AM as a major defensive cell for the purpose of clearance of particles from the lung. A significant increase in the activities of acid phosphatase (AP) and lactate dehydrogenase (LDH) was observed in cell free lavage fluid throughout the period of exposure. The maximum increase in their activities was observed on day 8th of exposure. Increased activities of these enzymes could be correlated with the degree of cytotoxic capability of UF-TiO$_2$.

A significant generation of hydrogen peroxide (H$_2$O$_2$) in AM was observed on day 8th and 16th of exposure. The overloading of UF-TiO$_2$ may activate a respiratory burst mechanism, leading to an increased production of H$_2$O$_2$. A significant and progressive increase in lipid peroxidation (LPO) was observed at different time intervals. The addition of NADPH and iron greatly enhanced the production of LPO in the exposed AM. The release of ROS could oxidize polyunsaturated fatty acid located in the plasma membrane resulting in the formation of breakdown product thiobarbituric acid reactive substances (TBARS) of LPO.
Summary and conclusions

A significant increase in the activities of glutathione redox enzymes in AM was observed. The maximum increase in the activities of glutathione peroxidase (GPx), glucose 6-phosphate dehydrogenase (G6PD) and glutathione reductase (GR) was 50%, 55%, 40% respectively on day 8th. Whereas increase in the activity of glutathione S-transferase (GST) was maximum on day 16th (66%). The increased activities of these enzymes showed an adaptive response against oxidative stress.

While a significant and progressive depletion in the level of GSH was observed, the maximum decrease in GSH content was on day 16 in UF-TiO$_2$ exposed rats. A decrease in ascorbic acid was also observed with maximum on day 16. GSH and ascorbic acid are important soluble antioxidants of mammalian lung defense and the decrease in the level further indicates oxidative stress.

In conclusion the results indicate that these alteration in cellular enzymatic and non-enzymatic balance could not diminish the enhanced LPO and the increased rate of H$_2$O$_2$ generation, suggesting that UF-TiO$_2$ exposure may have posed increased toxicity and oxidative stress in AM which may eventually, have led to pulmonary pathological changes.

2. Activation of alveolar macrophages and peripheral red blood cells in response to toxic fiber/particle

The cytotoxic and oxidative responses of these fibers/particles were measured by a single intratracheal exposure at one time point when inflammatory and fibrotic reactions are in progress. Alveolar macrophages (AM) and red blood cells (RBC) were choosen to study the toxic potential of two types of mineral fibers viz. crocidolite and chrysotile and a mineral particle UF- TiO$_2$. An increase in the number of AM population, followed by an increase in the activities of AP and LDH was noticed. The maximum increase was with crocidolite followed by chrysotile and minimum with UF-TiO$_2$. The increase in AM population indicated defensive mechanism and an
increase activities in AP and LDH, indicate damage to AM membrane. An increase in protein content in cell free lung lavage fluid may be due to leakage of internal constituents from the injured AM.

A significant increase in LPO in AM and RBC was observed. An increase in \( \text{H}_2\text{O}_2 \) generation in AM was also noticed. These indicate oxidative stress due to excessive production of ROS.

Alterations in the activities of antioxidant enzymes were observed both in AM and RBC of exposed animals. A significant inhibition in the activities of GPx and GR in AM was observed with crocidolite and chrysotile which was non-significant in case of UF-TiO\(_2\). The activities of GPx and catalase were also inhibited in RBC with these mineral fibers/particles. In the present studies the significant inflammatory responses seen in the RBC reflected the same order of severity as those seen in AM and cell free lung lavage fluid: crocidolite > chrysotile > UF-TiO\(_2\).

A depletion in soluble antioxidants namely GSH and ascorbic was also noticed in both AM and RBC. The depletion in their levels followed the similar pattern. Both GSH and ascorbic acid function directly in the destruction of ROS and their lower level indicates oxidative stress. The decreased level of GSH in RBC may be due to excessive release of ROS by AM in lung tissue which consumes GSH directly or indirectly via LPO. One month after exposure to mineral fibers the first cellular response is the accumulation of inflammatory cells at site of fiber deposition. Further an inflammatory cell reaction, accumulation of myofibroblasts and increased volume of interstitial matrix was also observed. In the case of UF-TiO\(_2\) the inflammatory response as observed by histopathological changes appears to be mild.

The most significant finding of the present study is the toxic response of fibers/particles in the order of crocidolite > chrysotile > UF-TiO\(_2\) in both the systems.
Summary and conclusions

Crocidolite, the most toxic and known carcinogenic fiber induces maximum toxicity and oxidative stress followed by chrysotile although the differences were not significant. On the other hand, in comparison to both the mineral fibers, UF-TiO₂ induces far less toxicity and oxidative stress.

3. Chrysotile fiber-mediated imbalance in the glutathione redox system in the development of pulmonary injury

Rats were exposed to a mineral fiber namely chrysotile by a single intratracheal instillation (5 mg/rat). After 1, 4, 8, 16, 30, 90, and 150 days of exposure, rats were assessed biochemically to ascertain the status of oxidative stress. At the initial stages of exposure when cytotoxic and AM defensive reactions have taken place, a gradual and progressive depletion in GSH level of AM was observed. This decrease in the GSH content is an early event in inflammatory response. A slight decrease in blood GSH was also observed. The depletion of GSH in lung cytosol started from day 16 and reaches maximum on day 150. The depletion in the GSH content may be one of the causative factor for the development of mineral fiber-induced pulmonary injury and cell proliferation. A depletion in ascorbic acid, a soluble antioxidant, was also observed throughout the period of exposure in lung cytosolic fractions.

A significant increase in the activities of GPx, GR, and G6PD in the cytosolic fraction of the lung after chrysotile exposure was observed. The maximum induction in GPx was observed at day 90 (133%) and day 150 (129%). GR activity was significantly induced at all time intervals with a maximum on day 4 (103%). A gradual and progressive increase in the activity of G6PD was observed which reached maximum on day 150. The increased activity of GPx may be due to fast removal of ROS. Induction in the activities of GR and G6PD appear to be a defensive metabolic adaptation to mammalian GSH pool during initiation and progression of asbestosis. A decrease in the activity of GST may further deteriorate the situation.
An increase in LPO was observed, which was significant and persisted throughout the period of exposure. This increase in LPO indicates oxidative stress imposed by chrysotile fibers.

Thus it may be concluded from the present study that the decrease in cellular GSH may serve as an early indicator of lung diseases, whereas increased LPO and enzymatic changes may give an insight into the proliferative and asbestotic stage of diseased animals.

4. N-acetylcysteine attenuates oxidant-mediated toxicity induced by chrysotile fibers

Induction of oxidative stress by asbestos is one of the major cause of its pathogenicity as reported by several authors and also in the present study. Further, a significant and progressive increase in $\text{H}_2\text{O}_2$ generation and TBARS was also observed. This indicate oxidative stress imposed by these fiber. Depletion in the level of GSH and alteration in the activities of antioxidant enzymes i.e., GPx, GR and G6PD regulating oxidative tone indicate oxidative stress.

A study was, therefore, initiated by supplementing N-acetylcysteine (NAC) a precursor of GSH to see its effect on asbestos induced oxidative stress. The animals exposed to chrysotile received 50mg NAC/Kg body weight by ip route daily and were sacrificed after 1,4,8 and 16 days. It was observed that administration of NAC in chrysotile exposed animals caused a significant decrease in $\text{H}_2\text{O}_2$ generation on day 8th (22%) and day 16th (26%) and decreased in the production of TBARS in cellular fraction on day 8th (24%) and 16th (31%) and in acellular fraction on day 16th (27%) in comparison to chrysotile exposed animals. A significant recovery in GSH content was observed after administration of NAC in both the system at a latter stage. After administration of NAC to chrysotile exposed animals the activity of GSH redox enzymes showed a trends towards attaining normal basal activity. In this preliminary
study NAC may be protecting the cells against oxidative damage induced by chrysotile fibers. This protection may be due to its ability to maintain intracellular GSH level/oxidant scavenging capability. Further detailed studies in this direction are needed.

5. **Prolonged toxicity in experimental animals exposed to crocidolite and chrysotile and mineral particle ultrafine titanium dioxide**

After seven months of a single intratracheal instillation with mineral fibers viz. crocidolite and chrysotile and mineral particle UF-TiO\(_2\) to rats, an increased lung weight indicating fibrosis was observed. The level of collagen, sialic acid and hexosamine content was found to increase maximum with crocidolite followed by chrysotile and minimum with UF-TiO\(_2\). The accumulation of collagen indicates that the fibrosis has set in which may eventually harm the functional activities of the lung. Both sialic acid and hexosamine a mucopolysaccride were also increased which also support the development of pulmonary fibrosis. A significant increase in TBARS was observed in lung microsomes and red blood cells of exposed animals. The enhancement of LPO indicated increased oxidative stress.

A decrease in the level of soluble antioxidants namely GSH and ascorbic acid with mineral fibers/particles in lung cytosol and RBC was observed. The decrease was more pronounced in lung cytosol with maximum with crocidolite followed by chrysotile and minimum with UF-TiO\(_2\). The decrease in the level of ascorbic acid followed the similar pattern. Thus, the significant depletion in the levels of these antioxidant in the fibrotic lungs suggest that the mineral fibers/particles-induced oxidative stress, damage the antioxidant defense of the lung and the lung fails to protect itself against the development of fibrosis.
An alteration in primary and secondary antioxidant enzymes in the lung cytosol and RBC of exposed animals was observed. A significant increase in the activities of GPx, GR and G6PD was observed in the lung cytosol compared to their respective control. The observed increase in the activities of GPx, GR and G6PD may be defensive and an adaptive response due to burden by mineral fibers/particles. An increase in the activity of G6PD and decrease activity of catalase was noticed in RBC. The pattern of increase/decrease was maximum with crocidolite followed by chrysotile and UF-TiO2. These alteration in antioxidant enzymes in RBC reflected the exaggerated production of ROS in the lung.

An alteration in pulmonary drug metabolizing enzymes in lung microsomes and cytosolic fractions of exposed animals was also observed as compared to control. A significant increase in the activities of epoxide hydrolase (EH), Benzo(a) pyrene hydroxylase and cytochrome P-450 content were observed with mineral fibers and comparatively less with UF-TiO2 particle. The induction of EH, benzo (a) pyrene hydroxylase and cytochrome P-450 may further aggravate the situation. This may produce more reactive metabolites in the target tissue and, therefore, increase the possibility of higher adduct formation with the biological macromolecules, while a significant decrease in the activity of GST was observed only with crocidolite and chrysotile. The decrease in the activity of GST may in turn result in the retention of various lipophilic carcinogens in the lung which may additionally be involved in the carcinogenic response induced by mineral fibers/particles.

It may be concluded from the present study that in comparison to mineral fibers namely crocidolite and chrysotile, UF-TiO2 particles are less fibrogenic, induce
Summary and conclusions

less oxidative stress and alter the xenobiotic metabolizing enzyme system of the lung to a lesser extent.

Conclusions

These studies suggest that the mineral fibers/particles, produce oxidative tone resulting in enhanced oxidative stress in the system. The result further indicate that GSH depletion and alterations in GSH redox enzyme system plays a crucial role in the toxicity of mineral fibers. Nutritional supplementation of N-acetylcysteine a precursors of GSH may help exposed population against toxic manifestation of mineral fibers.