



Journal of Medical Sciences

ISSN 1682-4474

science
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JMS (ISSN 1682-4474) is an International, peer-reviewed scientific journal that publishes original article in experimental & clinical medicine and related disciplines such as molecular biology, biochemistry, genetics, biophysics, bio-and medical technology. JMS is issued eight times per year on paper and in electronic format.

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Pulmonary Toxicity of Copper Oxide (CuO) Nanoparticles in Rats

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The present study was aimed to evaluate the ability of the Copper Oxide nanoparticles to induce pulmonary toxicity in rats following Intra-Tracheal (IT) instillation. The lungs of rats were intra-tracheally instilled with Phosphate-Buffered Saline (PBS) or CuO nanoparticles or quartz silica particles at a dose of 1 and 5 mg kg⁻¹ body weight. Following exposure, bronchoalveolar lavage (BAL) fluid and lungs etc. were collected at 1 day and one week of post instillation of nanoparticles. Bronchoalveolar Lavage (BAL) fluid analyzed for Lactate Dehydrogenase (LDH), Alkaline Phosphatase (ALP), Total proteins to assess the pulmonary toxicity. CuO and quartz exposed rats produced a transient dose dependant increase in BAL fluid ALP, LDH, Total Proteins and Total leucocytes count than control after 1 day and 1 week post exposure periods. Lung tissue homogenate is made with Phosphate Buffer Saline (PBS) and antioxidants like Super Oxide Dismutase (SOD), Catalase were estimated using tissue homogenate. It was observed a dose dependant decrease in SOD, catalase values in exposed rats than control at all post exposure periods. The histopathological examination of lungs revealed a dose-dependent degeneration, fibrosis and granuloma formation in nanoparticles exposed rats 1 day and were worsen at 1 week post-instillation periods. These results suggest the instillation of CuO nanoparticles produced a greater pulmonary toxicity in rats. The lung tissue homogenate revealed the reduction of antioxidant capacity of rats following exposure of nanoparticles. In conclusion, the present study results revealed the pulmonary toxicity of CuO nanoparticles in rats following their exposure.

Key words: Nanoparticles, intra-tracheal instillation, CuO, Quartz, BAL fluid, LDH, ALP, lung tissue homogenate, SOD, catalase, histopathology, pulmonary toxicity

INTRODUCTION

Inhalation and intratracheal instillation studies have been conducted in rats to assess the toxicity of various metal oxide nanoparticles in rats following their exposure, metal oxide nanoparticles have inflammatory potentials that leads to inflammation, fibrosis, pulmonary granuloma formation and oxidative stress (Oberdorster *et al.*, 2005). Metal oxide nanoparticles are widely used in manufacturing of commercial products and their industrial applications are expanded. Silica (SiO₂) is one of the most abundant oxides present in ambient air and is in crystalline (quartz) or amorphous form and is widely used in many applications such as fillers in the rubber industry, anti-caking agents in powder materials (Merget *et al.*, 2002). Copper oxide (CuO) nanoparticles are used in antimicrobial preparations, heat transfer fluids, semiconductors or intrauterine contraceptive devices (Aruoja *et al.*, 2009). Oral administration of copper oxide nanoparticles induces hepatotoxicity and nephrotoxicity in exposed rats (Lei *et al.*, 2008) it is well known that this toxicity is mediated by the generation of oxidative stress in the liver and the kidney tissues. In the present study, we have evaluated the pulmonary toxic effects of Copper Oxide (CuO) nanoparticles in rats following their exposure.

MATERIALS AND METHODS

Materials: The Test CuO Nanomaterials (size<50nm, surface area 29 m² g⁻¹, crystalline shape, diameter <50 nm, length <50 nm) were obtained from Sigma Aldrich, St. Louis, USA.

Experimental animals: Adult male wistar albino rats (Mahaveer Enterprises, Hyderabad, India) of 8 weeks old at study start (Mean weight in the range of 180-200 g) were selected and housed in polypropylene cages in a room where the congenial temperature is 27±1°C and 12 h light and dark cycles were maintained. The animals were allowed to acclimatize to the environment for 7 days and supplied with a standard pellet diet and water *ad libitum*.

Preparation of nanoparticle suspensions: Fine particles of CuO nanoparticles are slightly soluble in water. For instillation into rat lungs they were dispersed in non toxic dispersing vehicle. All the nanoparticles suspensions were prepared in PBS 1% at a concentration of 10 mg mL⁻¹ by briefly shearing (2 min in a small glass homogenizing tube) and subsequently sonicated (1-2 min) nanoparticle samples. All samples with different concentrations were re-sonicated on the day of dosing before instillation. Each sample was vortexed just before instillation.

Intratracheal instillation: The rats were anesthetized with 3-5% ether in a small chamber and individual rats were

secured on an inclined plastic platform and anesthetization is continued via a small nose cone. The trachea was exposed by a 1-cm incision on the ventral neck skin for instillation of the nanoparticle suspension. The intratracheal fast instillation procedure for rats used by, modified to ensure that the instilled material might be delivered into the lungs of rats with a good distribution (Shilpa and Anreddy, 2012; Lam *et al.*, 2004).

Study design: A total of 40 male wistar rats were used in the study and were divided into 5 groups of 4 in each group per each interval up to 2 intervals. Groups of rats were instilled intratracheally with single dose of 1 or 5 mg kg⁻¹ of CuO nanoparticles, quartz-crystalline silica particles (Q). All particles are prepared in a volume of PBS (1.0%). Group of PBS (1%) and quartz served as solvent and positive control, respectively:

- Group 1:** Served as solvent control and treated with 1 BS
- Group 2:** Receives CuO Nanoparticles (1 mg kg⁻¹) (Test group)
- Group 3:** Receives CuO Nanoparticles (5 mg kg⁻¹) (Test group)
- Group 4:** Receives Quartz (1 mg kg⁻¹) (Positive control)
- Group 5:** Receives Quartz (5 mg kg⁻¹) (Positive control)

Collection of BAL (Bronchoalveolar lavage) fluid: BAL fluid was collected from all the sham and particulate exposed rat lungs at 1 day and 7 days of post exposure periods. For the collection of BAL fluid, warm PBS solution was used for lavaging BAL fluid from lungs of sham and particulate-exposed rats using reported methods (Warheit *et al.*, 2005). About 8 mL of PBS was used to fill the lungs per wash. Gentle manipulation was done after insertion of the PBS and during the withdrawal of lavage fluid. BAL fluid analysis was done with the first recovered 12 mL of lavaged fluids.

Analysis of BAL fluid: From BAL fluids, biochemical assays like estimation of Lactate dehydrogenase (LDH) (using Erba diagnostics kit, Germany), alkaline phosphatase (ALP) (using Excel diagnostics kit, Hyderabad, India), total protein (using Excel diagnostics kit, Hyderabad, India) concentration were measured using respective diagnostic kits. Lactate dehydrogenase is a cytoplasmic enzyme and its increased levels used as an indicator of cell injury. Alkaline phosphatase is an enzyme, which is a measure of type II alveolar epithelial cell secretory activity and its increased levels in BAL fluids is considered to be an indicator of type II cell toxicity. Increases in BAL fluid protein concentrations generally are consistent with enhanced permeability of vascular proteins into alveolar regions. Total leucocytes count was performed manually using Neuber counting chamber, this count is an indicator of inflammation.

Collection of Lungs and Preparation of Lung Tissue Homogenate:

After 24 h and 1-week of post-instillation of nanoparticles, the animals were sacrificed by giving high dose of ether anesthesia and then lungs were removed from all the animals and are washed with prechilled physical saline, homogenized with prechilled saline in tissue homogenizer and centrifuged at 3000 rpm for 10 min at 40°C. The supernatant was collected and assayed for the estimation of Catalase (Beers *et al.*, 1952) and Superoxide dismutase (SOD) activity (Misra and Fridowich, 1977).

Histopathological examinations: The lungs of rats exposed to particulate-exposed or PBS controls were prepared for microscopy by airway infusion under pressure (21 cm H₂O) at 24 h, 1 week and 1 month. Sagittal sections of the left and right lungs were made with a razor blade. Tissue blocks were dissected from left, right upper and right lower regions of the lung and were subsequently prepared for light microscopy (paraffin embedded, sectioned and hematoxylin-eosin stained) and evaluated.

Statistical analysis: All the experimental values expressed as mean±SD and were compared with sham control value at each time point. One-way analysis of variance (ANOVA) and Dennett test were used to compare means from the control group and each of the groups exposed to particulates and the statistical significance is judged at the 0.05 probability level.

RESULTS

The pulmonary toxicity of CuO nanoparticles was assessed by analysis of BAL fluid for tissue damage markers of LDH and ALP and the results were expressed in Fig. 1 and 2, respectively. Exposure of CuO nanoparticles to rat lungs resulted in a transient dose-dependent ($p<0.05$) increase in BAL fluid LDH and ALP. The results of total proteins and leucocytes count in BAL fluid of rats exposed to nanoparticles were showed in Fig. 3 and 4, respectively.

Analysis of rat lung tissue homogenate: Lungs tissue homogenate of rats of sham control and particle exposed rats were analysed for catalase, SOD activity to estimate the anti oxidant capacity in rats following exposure of nanoparticles and the results were showed in Fig. 5 and 6 respectively. It was observed a dose dependant decrease in SOD, catalase values in exposed rats than control at all post exposure periods ($p<0.05$).

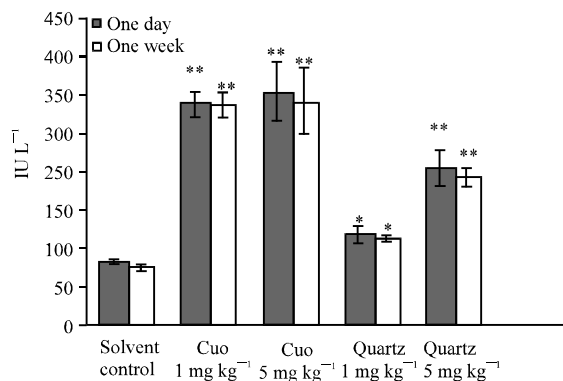


Fig. 1: LDH (IU L⁻¹) levels in BAL fluids of rats exposed to CuO and quartz particles, All the values are expressed as mean±SD; * $p<0.05$, ** $p<0.01$

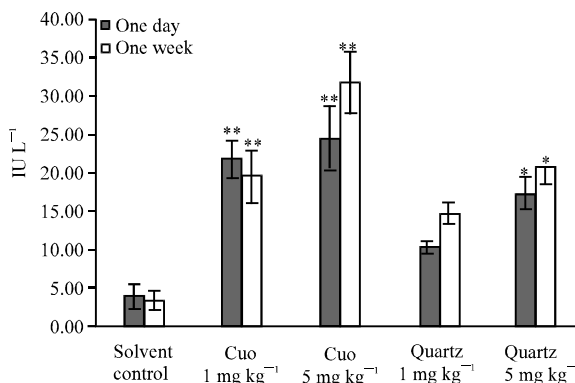


Fig. 2: ALP levels in BAL fluids of rats exposed to CuO, Quartz particles, All the values are expressed as mean±SD; * $p<0.05$, ** $p<0.01$

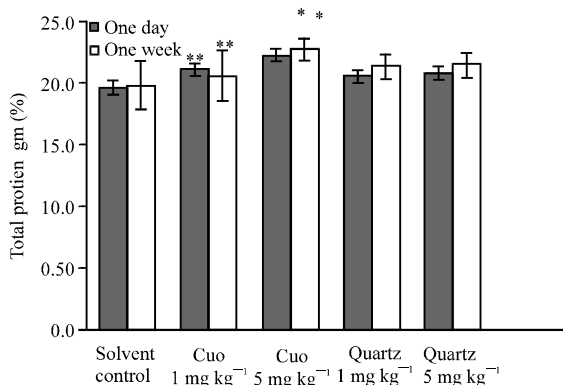


Fig. 3: Total Protein (gm %) levels in BAL fluids of rats exposed to CuO and quartz particles, All the values are expressed as mean±SD; * $p<0.05$, ** $p<0.01$

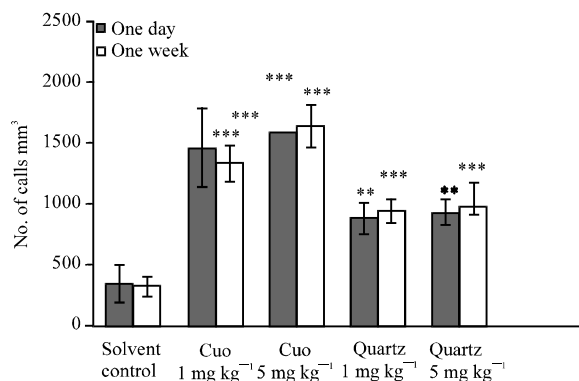


Fig. 4: BAL fluid Total Leucocyte count (cells mm⁻³) values in rats exposed to CuO nanoparticles and quartz particles, All the values are expressed as Mean±SD; *p<0.05, **p<0.01 and ***p<0.001

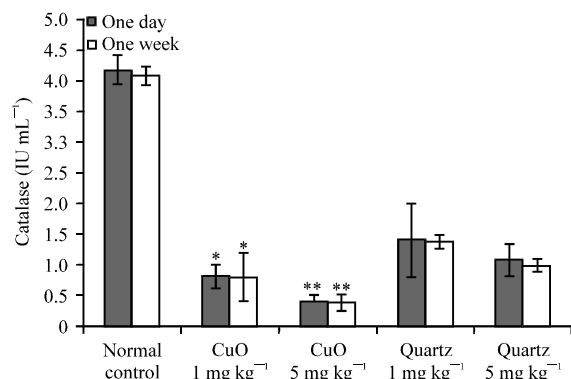


Fig. 5: Catalase (IU mL⁻¹) levels in lung tissue homogenate of rats exposed to CuO nanoparticles and quartz particles, All the values are expressed as mean±SD; *p<0.05, **p<0.01

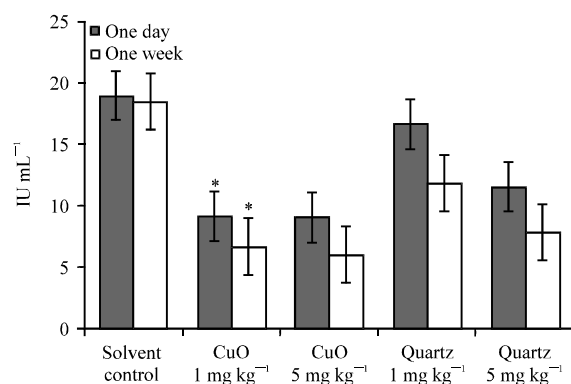


Fig. 6: SOD levels (IU mL⁻¹) in Lung tissue homogenate of rats exposed to CuO nanoparticles and Quartz particles, All the values are expressed as Mean±SD; *p<0.05

Histopathological analysis: Lung of normal control group rat did not reveal any histopathological changes (Fig. 7 a). Quartz silica particles exposed lungs at 1 and 5 mg kg⁻¹ after 24 h showed moderate congestion, hemorrhage, edema and presence of mononuclear cell infiltrations around bronchus and presence of fibrous tissue formation (Fig. 7d and e). Copper oxide group (a single dose of 1 and 5 mg kg⁻¹ 24 h) showed acute inflammatory reaction with infiltration of inflammatory cells in the alveoli, vacuolation, bullae formation. All this alterations indicates tissue damage in lung tissue (Fig. 7b and c). These changes were milder in quartz silica exposed lung at 1 and 5 mg kg⁻¹ after 24 h.

Quartz silica particles exposed lungs at 1 and 5 mg kg⁻¹ for 1 week showed severe congestion and rupture of alveoli, few mononuclear cell infiltrations and lymphoid aggregation (Fig. 8d and e). Copper oxide nanoparticles exposed lung at 1 and 5 mg kg⁻¹ for 1 week showed infiltration of mononuclear cells, granulomas and lymphoid aggregates (Fig. 8b and c). These changes were more severe when compared to the lungs of rats exposed to quartz silica nanoparticles for 1 week.

All these results indicating that copper oxide nanoparticles have inflammatory potentials that lead to irreversible chronic lesions such as fibrosis or granulomas.

DISCUSSION

In the present study, we have investigated the acute pulmonary toxic effects of copper oxide nanoparticles in rats following their exposure. We have also assessed the antioxidant status upon CuO nanoparticle exposure in rats following intratracheal instillation at two post exposure periods and to compare these toxic effects with those of quartz particles.

This study investigated that, upon exposure to all the mentioned particles at different doses, no mortality was produced in particle exposed rats. Results from the analysis of BAL fluid for tissue damage biomarker (LDH and ALP enzymes) levels revealed that, CuO nanoparticle and Quartz exposed rats (1 and 5 mg kg⁻¹) produced dose dependant acute lung toxicity and with significantly higher enzymatic levels (ALP, LDH) than in sham control at all the mentioned post exposure periods (Fig. 1, 2). From the results it was showed that there was dose dependant acute lung toxicity by intratracheal instillation of CuO nanoparticles in rats, supporting lung damage with CuO nanoparticle exposure.

Higher levels of total protein in BAL fluids obtained from the analysis of BAL fluid revealed that, CuO

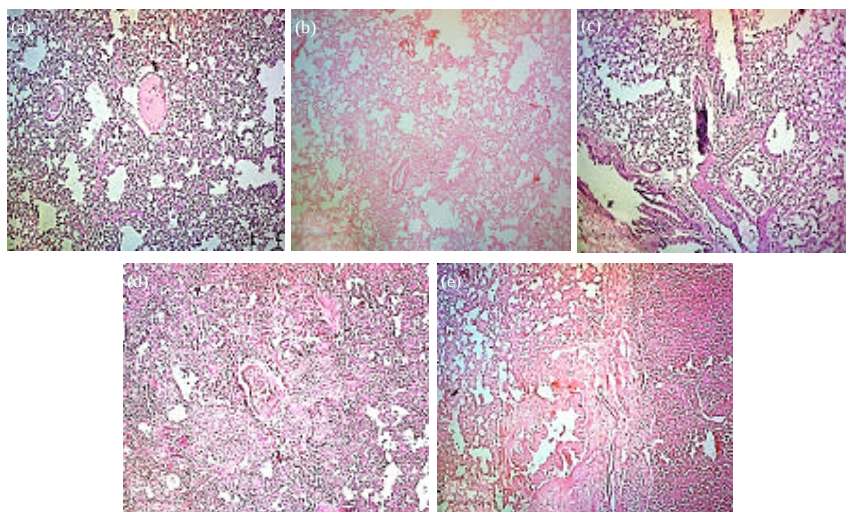


Fig. 7(a-e): Light micrograph of lung tissue from rat exposed to (a) PBS, (b) CuO (1 mg kg^{-1}), (c) CuO (5 mg kg^{-1}), (d) Quartz (1 mg kg^{-1}), (e) Quartz (5 mg kg^{-1}) at 24 h post-instillation exposure

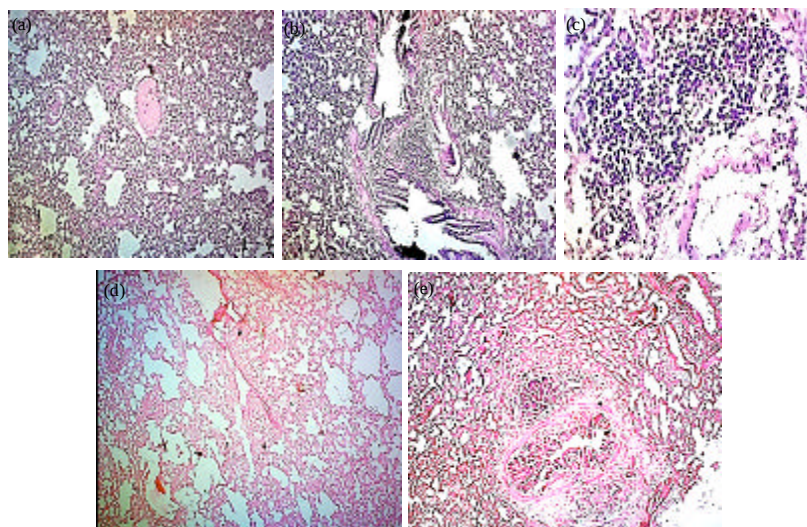


Fig. 8(a-e): Light micrograph of lung tissue from rat exposed to (a) PBS, (b) CuO (1 mg kg^{-1}), (c) CuO (5 mg kg^{-1}), (d) Quartz (1 mg kg^{-1}), (e) Quartz (5 mg kg^{-1}) at 1 week post-instillation exposure

nanoparticle and Quartz exposed rats (1 and 5 mg kg^{-1}) produced dose dependant acute lung toxicity and with significantly higher total protein levels than in sham control at all the mentioned post exposure periods. BAL fluid WBC/total leucocytes count was elevated in CuO exposed rats than Quartz exposed rats but it is not much significant.

Similar to the Quartz, CuO nanoparticle exposure showed the dose dependant infiltration of interstitial lymphocytes, lymphoid aggregation, acute inflammatory reaction with ruptured alveoli, mild edema, presence of

casts and few mononuclear cell infiltrations in the alveoli, vacuolation, bullae formation, fibrous tissue proliferation, granulomas and aggregation of lymphocytes in the form of nodule in the lungs of rats at 1 day post exposure period and were worsened at 7 day post instillation period. From the above results, it was suggested that, intratracheal instillation of these CuO nanoparticles produced a significant increase in pulmonary enzymatic levels, which was supported by histopathological examinations (Fig. 7, 8), indicating the pulmonary toxicity of CuO nanoparticles.

The results of the present study were supported with the results of Kim *et al.* (2011) who reported the effects of copper nanoparticle exposure on host defense in a murine pulmonary infection model. Copper nanoparticle exposure induced inflammatory responses with increased recruitment of total cells and neutrophils to the lungs as well as increased total protein and LDH activity in BAL fluid. Thus, exposure to Cu NPs may increase the risk of pulmonary infection.

The present results of this study were similar to our previous reports (Shilpa and Anreddy, 2012) and were similar with those of Lam *et al.* (2002, 2004), Warheit *et al.* (2004), Liu *et al.* (2004) and Morimoto *et al.* (2011). They reported that intratracheal instillation of different nanoparticles produced a dose dependent pulmonary toxicity in animals.

Antioxidants like SOD (Superoxide dismutase) and Catalase were estimated using lung tissue homogenate. The results showed that there was a dose dependant decrease in anti-oxidant (Catalase, SOD) potential in rats after 1 day and 1 week, post instillation of CuO nanoparticles, concluding that oxidative stress was induced in rats following exposure of CuO nanoparticles. These results were similar with our previous reports pertaining to the antioxidant capacity following exposure of nanoparticles (Kiranmai and Reddy, 2012).

It was reported that Oxidative stress has been implicated in the toxicity of nanoparticles and other studies suggested that excessive production of ROS and oxidative stress could be one of the possible mechanisms of NPs toxicity (Nel *et al.*, 2006; Ahamed *et al.*, 2011; Akhtar *et al.*, 2010a, b). Our results showed that CuO nanoparticles induced dose dependent toxicity and oxidative stress in rats. CuO nanoparticles induced the generation of ROS in exposed rats, antioxidants SOD and Catalase levels were significantly lower in CuO nanoparticles exposed rats. The depletion of SOD and Catalase, combined with the increased levels of ROS suggested that oxidative stress might be the primary mechanism for toxicity of CuO NPs in rats following their exposure. Moreover, our results are consistent with previous results suggesting that toxicity of CuO NPs mediated through ROS generation and oxidative stress (Griffitt *et al.*, 2007; Fahmy and Stephanian, 2009).

CONCLUSION

The present study results of both BAL fluid analysis and histopathological examinations of rat lungs exposed to copper oxide nanoparticles suggest the dose dependent pulmonary toxicity following their exposure. It is also observed the reduction of antioxidant capacity in rats following exposure of nanoparticles. These toxic

effects of nanoparticles were comparable with those of standard toxic nanoparticles, quartz.

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