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CHEMICAL AND BIOENGINEERING FOR SUSTAINABLE ENVIRONMENT

Melatonin ameliorates chronic copper-induced lung injury

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Abstract

Copper (Cu) is an important trace element required for several biological processes. The use of copper is increasing gradually in several applications. Previous studies suggest that excess levels of copper are attributed to induce oxidative stress and inflammation, mediating tissue damage. Inline, melatonin the hormone of darkness has been reported to exhibit various therapeutic effects including strong free radical scavenging properties and anti-inflammatory effects. However, its effects against pulmonary injury promoted by copper are not explored and remain unclear so far. Therefore, the present study was aimed to investigate the protective effect of melatonin against copper-induced lung damage. Female Sprague Dawley (SD) rats were exposed to 250 ppm of copper in drinking water for 16 weeks and treated with melatonin (i.p.) 5 and 10 mg/kg from the week (13–16th). The extent of tissue damage was assessed by tissue oxidative stress parameters, metal estimation and histological analysis. Copper-challenged rats showed altered oxidative stress variables. In addition, metal analysis revealed increased copper accumulation in the lungs and histological staining results further indicated severe tissue injury and inflammatory cell infiltration in copper-exposed rats. To this side, treatment with melatonin showed antioxidant and anti-inflammatory activities evidenced by reduced oxidative stress, tissue inflammation and collagen deposition as compared to copper-exposed animals. Moreover, spectral findings suggested melatonin treatment modulated the frequency sift, as compared to copper-challenged animals. Altogether, the present results suggest that melatonin might play a potential role in preventing copper-induced lung aberrations via inhibiting the ROS-mediated oxidative stress and inflammation.

Keywords Copper · Lungs · Melatonin · Oxidative stress · Inflammation

Introduction

Copper is an important trace element involved in various biological activities like antioxidant mechanism, neurotransmitter biosynthesis, mitochondrial respiration, heme synthesis and iron absorption and also plays a significant role as a cofactor for enzymes like superoxide dismutase-(SOD)-1, ceruloplasmin, lysyl oxidase and cytochrome c oxidase (Barceloux 1999; Kim et al. 2008; Lutsenko et al. 2019). Copper is absorbed into intestinal cells through copper transporter-(CTR)-1, 2 and is stored majorly in the liver, though it is highly concentrated in the brain, liver and kidney and is excreted mostly through bile. And the efflux of copper is encoded with ATP7A and ATP7B genes respectively (Gaetke and Chow 2003; Scheiber et al. 2013). Over accumulation of copper in the body leads to copper toxicity, through contaminated drinking water and fortuitous intake of copper-contaminated foods, resulting in several pathological conditions like Wilson's disease (WD), Menke's disease (MD), hemolytic anemia, gastrointestinal bleeding and diarrhoea (Scheiber et al. 2013). In addition, accumulated levels of copper are reported to involve in the progression of neurodegenerative diseases (Mathys and White 2017; Scheiber et al. 2013). Growing with the evidences, the increased use of copper as an additive in inks, skin products, cooking, semiconductor devices etc. promotes serious health issues. As during its application in several manufacturing processes,

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the metal particles get retained in the respiratory airways (Kim et al. 2019). Existing evidences have reported that the progressive deposition of copper in lungs is linked with time and dose, whereas liver is reported to be the most vulnerable organ against copper toxicity (Benson et al. 2000; Kumar et al. 2015; Patwa and Flora 2020; Zhang et al. 2021). In compliance, the deposition of copper in the lungs attains more focus as lungs are the major health concern of the population due to the inhaled particles, released from industries (Sebio et al. 2019). Recent evidences demonstrated that excess copper leads to alter various cellular and biological pathways inducing oxidative radicals (Janssen et al. 2018; Padrilah et al. 2017; Zhang et al. 2021). The generated oxidative radicals are characteristic of augmented tissue injury. Similarly, studies have found that copper could induce oxidative stress, autophagy and apoptosis via excessive generation of reactive oxygen species (ROS) (Liu et al. 2021; Zhang et al. 2021). ROS-mediated oxidative stress results in the initiation of several inflammatory responses (Gaetke et al. 2014; Yang et al. 2020). Researchers have also found that copper induces several pro-inflammatory cytokines like interleukin-(IL)-1β and tumor necrosis factor-(TNF)-α (Patwa and Flora 2020). The existing literature findings also suggest that copper acts as an anti-inflammatory agent but this certainly remains largely unexplained (Hussain et al. 2019; Walker and Keats 1976). Nevertheless, the stimulation of inflammatory responses is thought to be helpful against the invading foreign particles but the persistent release of inflammatory mediators governs the tissue injury via activating nuclear factor kappa B (NF-κB) pathway, inflammatory cytokines like TNF-α and IL-6, mitogen-activated protein kinases (MAPKs) and apoptosis (Gaetke et al. 2014). The transcription of immune and inflammatory response genes get involved in the metal toxicity including copper (Patwa and Flora 2020; Yang et al. 2020). Similarly, several preclinical studies have demonstrated that excessive generation of ROS damages lung tissue irreversibly (Benson et al. 2000; Zhang et al. 2021).

Pulmonary inflammation is a chronic disease that involves the permanent scarring of alveolar sacs, which eventually results in declined lung function (Ali et al. 2021b; Janssen et al. 2018; Padrilah et al. 2017). A recent study revealed that imbalanced copper homeostasis leads to generate ROS triggering tissue fibrosis (Janssen et al. 2018; Zhang et al. 2021). Nonetheless, diverse mechanisms are involved in the progression of pulmonary dysfunctions. In particular, the activated inflammatory responses stimulate pleiotropic factors like transforming growth factor (TGF-β), smad signalling and α-smooth muscle actin (α-SMA), including several other fibrotic markers, indicative of continuous exposure of copper that could be a cause of resultant pulmonary fibrosis (Lai et al. 2018). Moreover, lung injury is characterized by the excessive proliferation of extracellular matrix components in tissue matrix (Gérard et al. 2010; Wynn 2011). Another study reported the elevated levels of cytokines in the bronchoalveolar lavage fluid (BALF) after inhalation of copper nanoparticles which resulted in perivasculitis and alveolitis (Assad et al. 2018). However, to date, no efficient therapies are available to treat the respiratory dysfunctions. And the current available agents only provide symptomatic relief without reversing the pathological conditions. For instance, several metal chelators like D-penicillamine, triethylenetetramine (trien) and meso-2,3-dimercaptosuccinic acid (DMSA or succimer) are in effect to treat and chelate the metals from the body via increasing their excretion (Cao et al. 2015). And the reduction of oxidative stress and inflammation has been shown to ameliorate pulmonary injury and toxicity. In addition, earlier studies found that the use of antioxidants prevented the lung injury and pulmonary fibrosis (Ali et al. 2021a; Pooladanda et al. 2019).

Melatonin (N-acetyl-5-methoxytryptamine) is an amphiphilic tryptophan-derived indoleamine (Cipolla-Neto and Amaral 2018). Primarily, secreted from the pineal gland, apart from this, it is also secreted from various other sources like retina, gut, skin, platelets and bone marrow (Claustrat and Leston 2015). Melatonin is also known as "the hormone of darkness" as its synthesis and secretion are related to darkness during night time. The primary role of melatonin is to regulate the circadian rhythm; apart from this, it also exhibits various biological properties such as antioxidant, anti-inflammatory and free radical scavenger (Esposito et al. 2019; Hosseinzadeh et al. 2018). Various studies evidenced that melatonin shows anti-fibrotic effects on different organs like the heart, lung, liver and kidney by modulating various signalling cascades (Arslan et al. 2002; Che et al. 2020; Chen et al. 2011). In particular, its direct free radical scavenging property has been found as a key mechanism of protection. Additionally, Yu et al. demonstrated the anti-inflammatory effect of melatonin by maintaining a balance between pro- and anti-inflammatory cytokines (Yu et al. 2017). Melatonin treatment inhibited the fibrotic genes like fibronectin, TGF-β and connective tissue growth factor (CTGF) in diabetic cardiomyopathy mediated via NLRP3 inflammasome (Che et al. 2020). Several other studies have supported that melatonin exerts antioxidant properties by inhibiting lipid peroxidation in cells and overcome bleomycin (BLM)-induced pulmonary fibrosis (Arslan et al. 2002; Yildirim et al. 2006). To the best of our knowledge, no study has reported the beneficial effects of melatonin in animals challenged with copper. Therefore, the current study was designed to evaluate the protective effects of melatonin against tissue oxidative stress and inflammation in chronic copper-induced lung injury in rats.

Materials and methods

Chemicals and reagents

Copper sulphate, melatonin, sodium chloride, disodium hydrogen phosphate, sodium dodecyl sulphate, sodium hydroxide, sodium tartrate, acetic acid, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 2-thiobarbituric acid, perchloric acid, nitric acid, 1,1,3,3-tetramethoxypropane, L-glutathione, ammonium molybdate, hydrogen peroxide, bovine serum albumin (BSA), Folin-ciocalteu (FC) reagent, hematoxylin, eosin and ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of reagent grade.

Animals

Female Sprague Dawley (SD), 5–7-week-old rats were purchased from CSIR-Central Drug Research Institute, Lucknow, India. The animals were allowed to acclimatize to experimental conditions for 1 week before the start of the experiment. All the animals were treated humanely according to the experimental protocols and standards approved by the Institutional Animal Ethics Committee (IAEC), NIPER-Raebareli.

Experimental design

The female SD rats were divided randomly into five different groups ($n = 6$/group): a normal control group, copper-exposed group (250 ppm in R.O drinking water), copper-exposed + melatonin groups (5 mg/kg and 10 mg/kg) and melatonin group (10 mg/kg). Copper-exposed animals received 250 ppm of copper in R.O drinking water for 4 months. The doses of melatonin (5 mg/kg and 10 mg/kg) were prepared in absolute ethanol (5%), diluted with 0.9% normal saline and administered via intraperitoneal route (i.p.). The treatment with melatonin started after 3 months of copper exposure and continued for 1 month. Keeping in view the importance of dosing and time, we treated the animals with melatonin after 6 PM as more beneficial effects were observed during dark periods (Cipolla-Neto and Amaral 2018). All the animals were sacrificed after 4 months by administering urethane (Severs et al. 1981). Next, the lung tissue samples were collected and fixed in 4% formaldehyde solution for histological observation and the remaining tissue was stored at − 80 °C until required for various experimental protocols. A detailed graphical illustration of the experimental design is shown in Fig. 1.

Measurement of ceruloplasmin activity

The activity of ceruloplasmin was measured according to the previous protocol, with slight modifications (Patwa and Flora 2020). In brief, acetate buffer was added to the serum samples. Next, samples were incubated with O-dianisidine dihydrochloride for 15 min followed by the addition of H_2SO_4. Finally, the ceruloplasmin activity was measured at 540 nm.

Metal estimation

To estimate the levels of copper, lung tissue samples were processed as per the previous protocol (Patwa and Flora 2020). The concentration of copper in lung tissue was evaluated using inductively coupled plasma mass spectrometry (ICP-MS, PerkinElmer).

Preparation of tissue lysates for biochemical analyses

In short, 100 mg of lung tissue was homogenized in 1 mL of ice-cold phosphate-buffered saline (PBS) and the resultant homogenate was then centrifuged at 12,000 rpm for 10 min at 4 °C. Next, the supernatant was collected and various biochemical parameters such as malondialdehyde (MDA), glutathione (GSH), reactive oxygen species (ROS), catalase and protein content were evaluated accordingly.

Estimation of reactive oxygen species

The levels of ROS in the lung tissue were determined using 2,7-dichlorofluorescein diacetate (DCFDA) following previous protocol with slight modifications (Patwa and Flora 2020). The tissue supernatant (10 µL) was incubated with 5 µM DCFDA (5 µL) and PBS (985 µL) for 30 min at 37 °C. Next, the fluorescence was measured at excitation 485 nm/emission 529 nm. The obtained results were expressed as RFU/mg protein.

Estimation of thiobarbituric acid-reactive substance (TBARS)

The TBARS levels in lung tissue homogenate were estimated as per the previous method with some modifications (Ali et al. 2021b; Patwa and Flora 2020). The reaction mixture in the test tubes consisted of 750 µL thiobarbituric acid (0.8%), 100 µL tissue homogenate, 100 µL sodium dodecyl sulfate (8.1%) and 750 µL acetic acid (20%). The reaction mixture was incubated at 95 °C for 1 h and cooled under running tap water. The samples were centrifuged at 10,000 rpm for 10 min and the absorbance was measured at 532 nm. The TBARS levels were expressed as micromoles/mg protein.

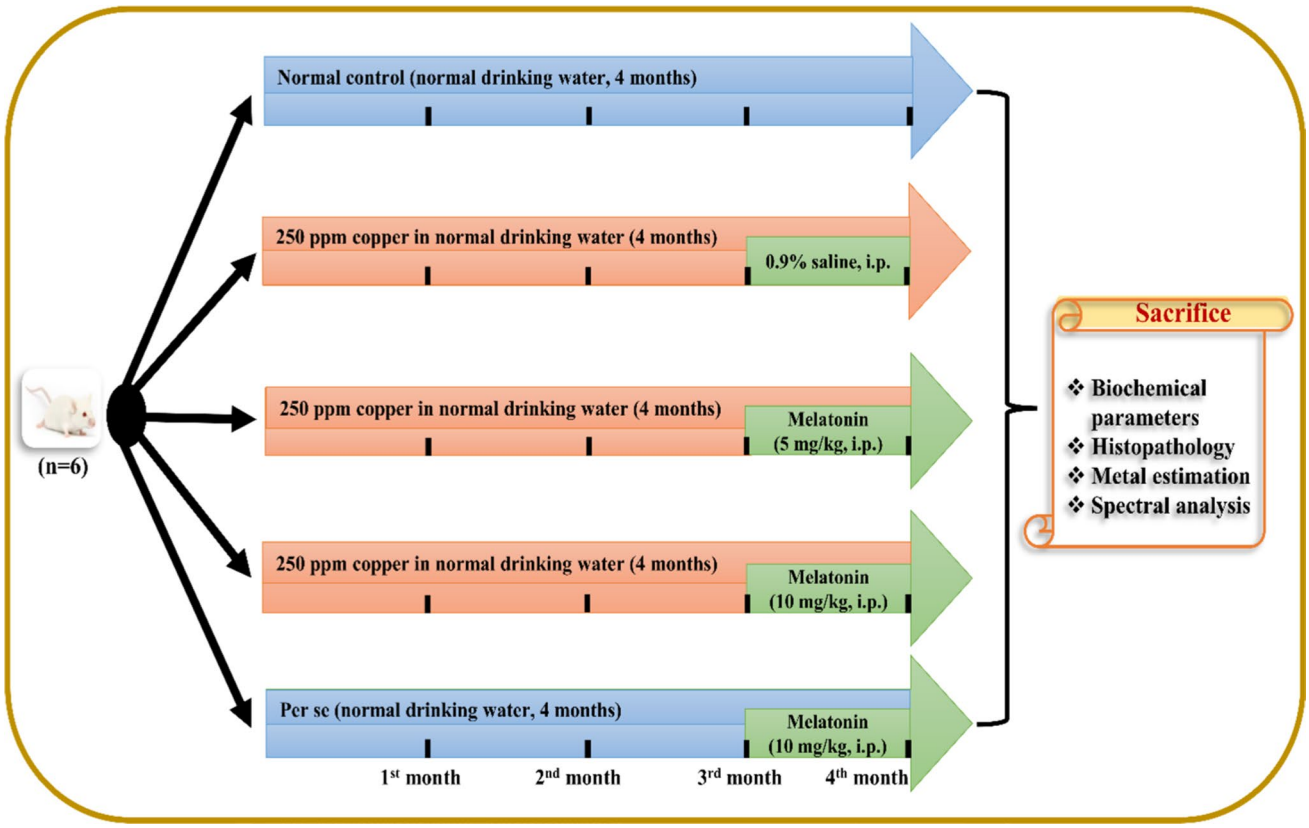

Fig. 1 Schematic illustration of the experimental design. Rats were exposed to 250 ppm of copper in R.O normal drinking water for 4 months and then the copper-challenged rats were treated with 5 and 10 mg/kg melatonin, i.p., following 3 months of copper exposure. The treatment with melatonin continued for 1 month. i.p., intraperitoneal; PPM, parts per million

Estimation of glutathione levels

The levels of glutathione (GSH) were determined using Ellman's method (Ali et al. 2021b). Tissue supernatant was incubated with Ellman's reagent (DTNB) at 37 °C for 10 min. The absorbance was measured at 412 nm and results were expressed as micromoles/mg protein.

Estimation of catalase activity

Catalase activity in lung tissue was estimated according to the previous protocol (Góth 1991). To quantify catalase activity, lung tissue supernatant was incubated with 65 mM H_2O_2 in 6.0 mM sodium potassium phosphate buffer, pH 7.4 for 1 min (sample 1). Assay control was set up with H_2O_2 and buffer (no enzyme control; blank 2) and buffer (no enzyme/no substrate, blank 3). The reaction was halted by the addition of ammonium molybdate (32.4 mM) to both the sample and the control mixtures. The absorbance was measured at 405 nm and the values were expressed relative to the concentration of protein.

Protein estimation

The total protein content in lung tissue was determined using Lowry method with slight modifications (Lowry et al. 1951). Five microliters of lung tissue supernatant was incubated in a reagent mixture containing 2% sodium carbonate, 1% copper sulfate and 2% sodium potassium tartrate for 10 min at 37 °C. Next, the FC reagent was added to the solution and incubated for 30 min at 37 °C. Finally, total protein content was measured at 660 nm using a multimode microplate reader (Synergy H1); BSA (0.2–1 mg/mL, range) was used as a standard.

Histopathological examinations

After the sacrifice, lung tissue was isolated and fixed in 10% formalin, dehydrated with a series of graded alcohol, embedded in paraffin wax and then sliced using a microtome. Haematoxylin and eosin (H&E), toluidine blue (TB) and picrosirius red (PSR) staining were performed as per the method described by Puebla-Osorio et al. (2017) and Vogel et al. (2015). For assessing

histopathological changes, 10 non-overlapping images/group were obtained. The H&E-stained sections were observed under light microscope to evaluate the histopathological alterations in the pulmonary tissue. Pulmonary tissue injury was defined by the thickening of the alveolar walls, tissue necrosis and inflammatory cell infiltration (Pooladanda et al. 2019; Zhou et al. 2018). The tissue injury and inflammatory cell infiltration were graded as follows: (1) tissue injury (0, no injury; 1, mild injury; 2, moderate injury; 3, severe injury; and 4, very severe injury), (2) inflammatory cell infiltration; (0, no cells; 1, few loosely arranged cells (< 10); 2, several cells around perivascular space (> 10- < 50); and 3, thick ring of inflammatory cells (> 50)). Sections were further stained with TB to evaluate the mast cell infiltration and the no. of purple color cells were counted in each group respectively. In addition, 5-µm thin lung tissue sections were stained with PSR to evaluate the degree of collagen deposition. Images were captured using a light microscope (Leica, Germany) and the % of area stained by PSR was quantified using ImageJ software (NIH, USA).

Spectrometric analysis

Fourier transform infrared (FTIR) spectroscopy is a powerful emerging technique used to identify functional groups and to monitor tissue damage. This technique offers data about the dynamics of proteins, lipids, carbohydrates and nucleic acids under different conditions (Zohdi et al. 2015). The soft tissues are susceptible to undergo changes in the functional groups due to heavy metal toxicity (Chouhan and Flora 2008). IR spectra of lung tissue samples were obtained at 4000–400 cm^{-1} using Bruker FTIR spectrometer (OPUS Version 7.5, USA).

Statistical analysis

All the statistical analysis was performed using GraphPad Prism 8 software. All results were expressed as the mean ± standard error of mean. Statistical differences amongst the groups were analysed by one-way ANOVA followed by multiple comparisons with Tukey's test. *P*-value < 0.05 was considered statistical significance.

Results

Effect of melatonin on body weight, lung index and lung weight

We observed no significant changes in the body weight of the animals in all the groups (Fig. 2A). And the average body weight of the rats was around 260–290 g. On the other side, we found that chronic copper administration induced the organ weight and organ index as compared to normal control. However, treatment with melatonin normalized the increased lung weight and lung index as compared to copper-exposed animals (Fig. 2B and C).

Effect of melatonin on copper levels in animals intoxicated with copper

As a result, increasing concerns with the contamination of water and applications of diverse metals in day-to-day life are a growing challenge on public health and are emerging as a leading cause for several diseases.

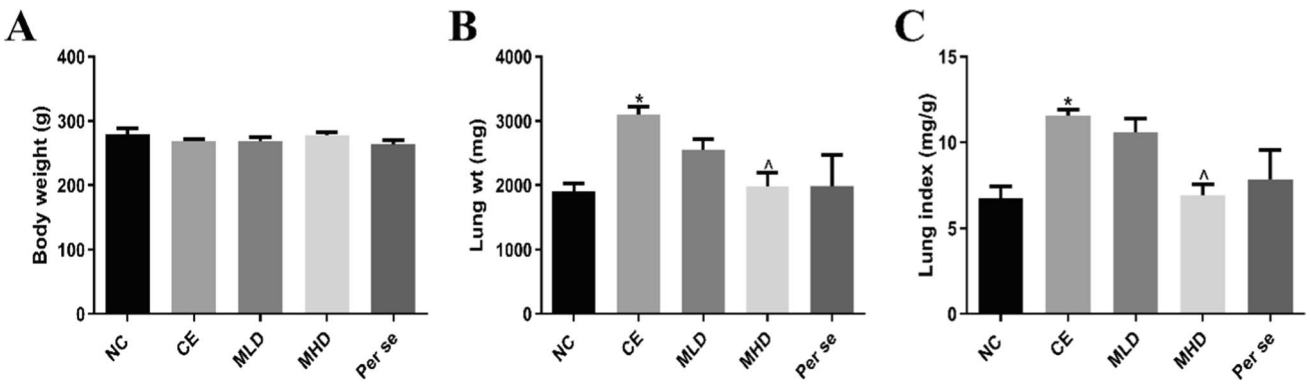

Fig. 2 Effect of melatonin on body and organ weight in copper-exposed rats. **A** Body weights. No changes in the body weight of the animals were observed in all the treated groups. **B**, **C** Lung weight and lung index. Melatonin significantly improved the lung weight and lung index altered by copper. Results are expressed as mean ± S.E.M. ($n = 6$). *$p < 0.05$ vs NC; ^$p < 0.05$ vs CE. NC, normal control; CE, copper exposed; MLD, copper + melatonin low dose (5 mg/kg); MHD, copper + melatonin high dose (10 mg/kg)

Hence, it is important to evaluate the deposition of metals in the tissue matrix. We observed that the levels of copper were increased slightly in animals exposed to copper as compared to normal control, while treatment with melatonin reduced the increased copper levels in lung tissue exposed to copper (Fig. 3A). Compelling with the above findings, we observed reduced levels of ceruloplasmin a major copper-carrying protein in copper-exposed animals as compared to normal control animals. In addition, treatment with melatonin increased the ceruloplasmin levels as compared to intoxicated animals (Fig. 3B). However, the changes were not significant in any of the groups, but still treatment with melatonin modulated the changes altered by copper.

Melatonin attenuates the tissue oxidative stress levels altered by copper intoxication

Oxidative stress is one of the common factors responsible for copper-induced pulmonary injury. The two major factors that lead to oxidative stress are elevated pro-oxidant and declined antioxidant levels. Here, we investigated two important pro-oxidant markers in the lungs, i.e. ROS and TBARS. It was observed that ROS levels were significantly

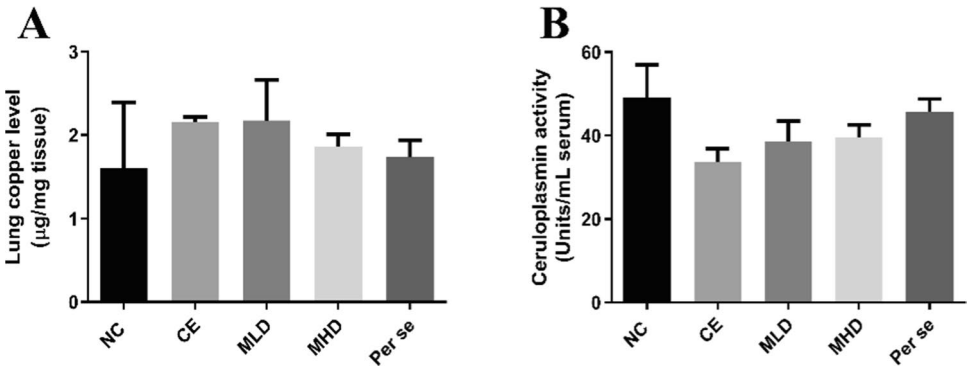

Fig. 3 Effect of melatonin on tissue copper levels. **A** Tissue copper levels. ICP-MS analysis evaluated reduced levels of copper in animals treated with melatonin. **B** Levels of ceruloplasmin. Copper exposure decreased the levels of major copper transporter. Interestingly, treatment with melatonin improved the levels of ceruloplasmin in copper-exposed animals. Results are expressed as mean ± S.E.M. ($n = 5$). NC, normal control; CE, copper exposed; MLD, copper + melatonin low dose (5 mg/kg); MHD, copper + melatonin high dose (10 mg/kg)

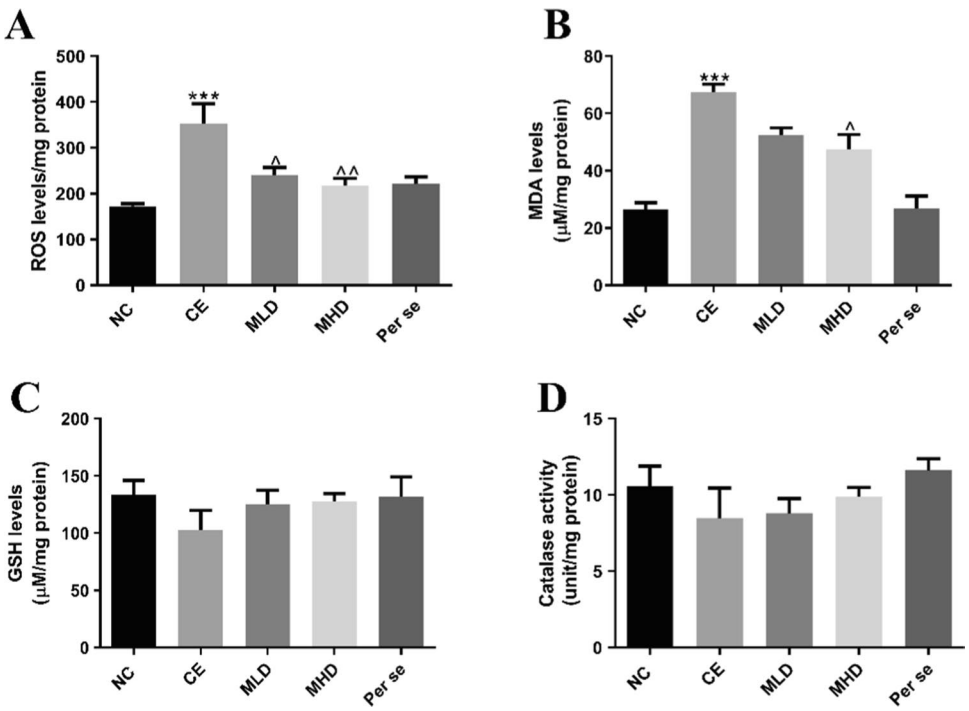

Fig. 4 Effect of melatonin on tissue oxidative stress markers. **A**, **B** Levels of ROS and MDA. Melatonin treatment significantly reduces the generation of ROS and lipid peroxidation induced by copper intoxication. **C**, **D** Levels of GSH and catalase. Copper treatment reduced the levels of endogenous antioxidants. However, treatment with melatonin partially augmented the levels of GSH and catalase in copper-challenged rats. Results are expressed as mean ± S.E.M. ($n = 6$). ***$p < 0.001$ vs NC; ^$p < 0.05$, ^^$p < 0.01$ vs CE. NC, normal control; CE, copper exposed; MLD, copper + melatonin low dose (5 mg/kg); MHD, copper + melatonin high dose (10 mg/kg)

increased in the copper-exposed group as compared to the normal control group. Nevertheless, treatment with melatonin dose-dependently reduced the generation of ROS as compared to copper-exposed animals (Fig. 4A). Similarly, elevated levels of ROS lead to enhanced lipid peroxidation in cells, which results in the increased levels of MDA, i.e. the final product of lipid peroxidation. We found that the levels of MDA were significantly high in copper-exposed animals as compared to the normal control. Treatment with melatonin significantly reduced MDA levels as compared to the copper-exposed group (Fig. 4B). However, melatonin alone–treated group did not exhibit any abnormal response when compared with normal control group, indicating the safety of the treatment.

Melatonin restores the antioxidant levels altered by copper

Under oxidative stress, the elevated level of ROS in the cells leads to lipid peroxidation which as a result centers to reduce the levels of endogenous antioxidants triggering tissue injury. Therefore, we estimated different antioxidant enzyme levels, like GSH and catalase. We observed that the levels of glutathione and catalase were decreased in the copper-exposed animals as compared to normal control animals. Similarly, treatment with melatonin had no significant effect on the levels of GSH and catalase (Fig. 4C and D). However, it restored the antioxidant balance altered by copper. These observations further confirmed the occurrence of pulmonary tissue injury might be associated with the imbalances in the oxidative stress and the potential role of melatonin in preventing tissue injury.

Effect of melatonin on chronic copper-induced histological changes and mast cell infiltration

The progression of lung injury involves a plethora of changes in the tissue architecture. Thus, to further confirm the role of melatonin in attenuating pulmonary injury, we performed histological analysis to study the effect of melatonin on the tissue injury and mast cell infiltration. H&E staining of the lung tissue sections revealed that lung tissue was significantly damaged, evaluated by the thickening of alveolar walls, interstitial edema and inflammatory cell infiltration in the copper-exposed animals. Nonetheless, treatment with melatonin significantly alleviated the

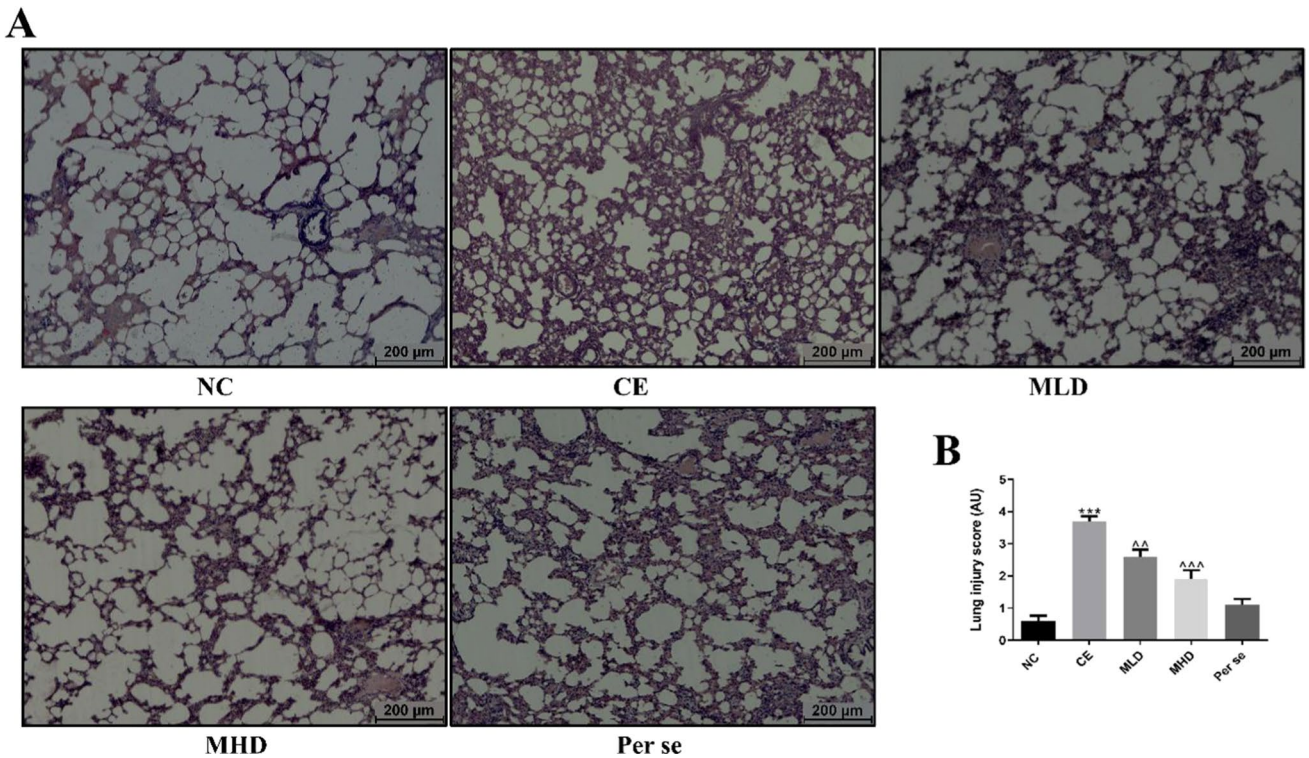

Fig. 5 Melatonin suppresses copper-induced histological alterations in pulmonary tissue. **A** Representative images stained with H&E demonstrated that melatonin restored copper-induced histological changes in the pulmonary tissue, including thickening of alveolar walls and interstitial edema. **B** Lung injury score. The obtained sections were used for quantitative analysis of the lung injury. Images were taken using an upright light microscope (100 × magnification, scale bar = 200 µm). Results are expressed as mean ± S.E.M. ($n = 3$). ***$p < 0.001$ vs NC; ^^$p < 0.01$, ^^^$p < 0.001$ vs CE. NC, normal control; CE, copper exposed; MLD, copper + melatonin low dose (5 mg/kg); MHD, copper + melatonin high dose (10 mg/kg)

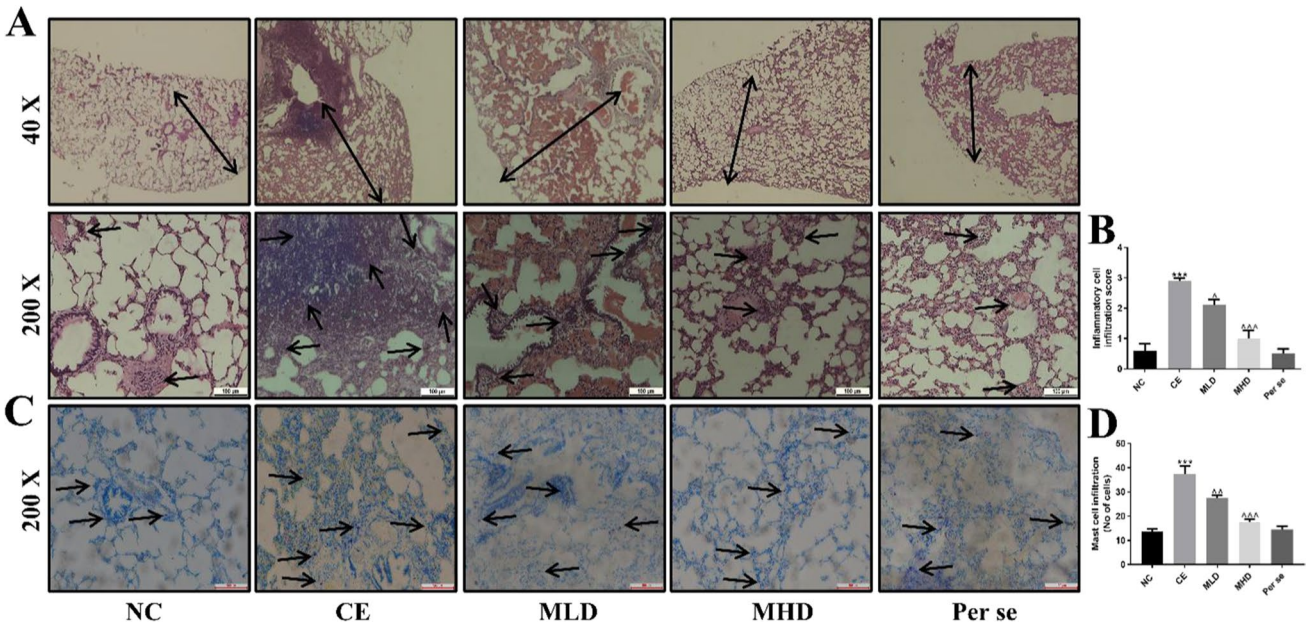

Fig. 6 Effect of melatonin on inflammatory and mast cell infiltration in animals challenged with copper. **A** 5-µm thin sections were stained with H&E and observed under light microscopy (40 × and 200 × magnification, scale bar = 100 µm). The infiltration of inflammatory cells was represented by the arrows (↑). Treatment with melatonin reduced the influx of inflammatory cells in copper-exposed animals. **B** Inflammatory cell infiltration score. **C** Representative images stained with toluidine blue evaluated mast cell infiltration (200 × magnification, scale bar = 100 µm). **D** Mast cell infiltration score showed (↑) reduced no. of mast cells in animals treated with melatonin. Results are expressed as mean ± S.E.M. ($n = 3$). ***$p < 0.001$ vs NC; ^$p < 0.05$, ^^$p < 0.01$, ^^^$p < 0.001$ vs CE. NC, normal control; CE, copper exposed; MLD, copper + melatonin low dose (5 mg/kg); MHD, copper + melatonin high dose (10 mg/kg)

copper-induced histological changes and infiltration of inflammatory cells (Figs. 5A and 6A). The histological scoring of the tissue injury and inflammatory cell infiltration showed reduced features of histological abnormalities in the treated groups as compared to copper intoxicated group (Figs. 5B and 6B). In addition, toluidine blue staining further revealed the significantly induced mast cell infiltration (purple color) in copper-exposed animals as compared to normal control animals. However, treatment with melatonin dose-dependently reduced the mast cell infiltration as compared to copper-exposed animals (Fig. 6C and D). The significant reversal of tissue injury and mast cell infiltration in copper-exposed animals suggested that melatonin reduces the pulmonary injury by inhibiting the process of tissue inflammation. On the other side, it is worth mentioning that melatonin alone–treated animals showed no abnormalities in the lung histoarchitecture, further suggestive of the safety of the treatment.

Melatonin suppressed collagen deposition in animals challenged with copper

Given the critical role of oxidative and inflammatory signalling in the regulation of tissue injury and deposition of extracellular matrix proteins, we then sought to assess the effect of melatonin on collagen deposition and tissue remodelling in copper-challenged rats. Our results showed that collagen deposition (red color) was significantly induced in copper-exposed animals than normal control animals, visualized by PSR staining. To this end, treatment with melatonin diminished the excessive deposition of collagen and tissue injury induced by copper (Fig. 7A and B).

Effect of melatonin on the hydroxyl functional group changes altered by copper; FT-IR analysis

Finally, we investigated the role of melatonin on sift in functional groups. For this, spectral analysis of lung tissue was performed. IR spectra showed number of peaks in the characteristic 4000–400 cm^{-1} IR regions. We found that the hydroxyl group peak at 3285.81 cm^{-1} in the normal control group (Fig. 8A), whereas this peak shifted towards lower frequency IR region at 3277.36 cm^{-1} in the copper-exposed group (Fig. 8B). Interestingly, we observed that treatment with melatonin dose-dependently shifted the peaks to 3280.11 cm^{-1} (5 mg/kg) and 3284.19 cm^{-1} (10 mg/kg) as compared to copper-exposed animals (Fig. 8C and D). Moreover, melatonin alone–treated group did not show any atypical response as compared to normal control animals and the peak was observed at 3282.53 cm^{-1} (Fig. 8E).

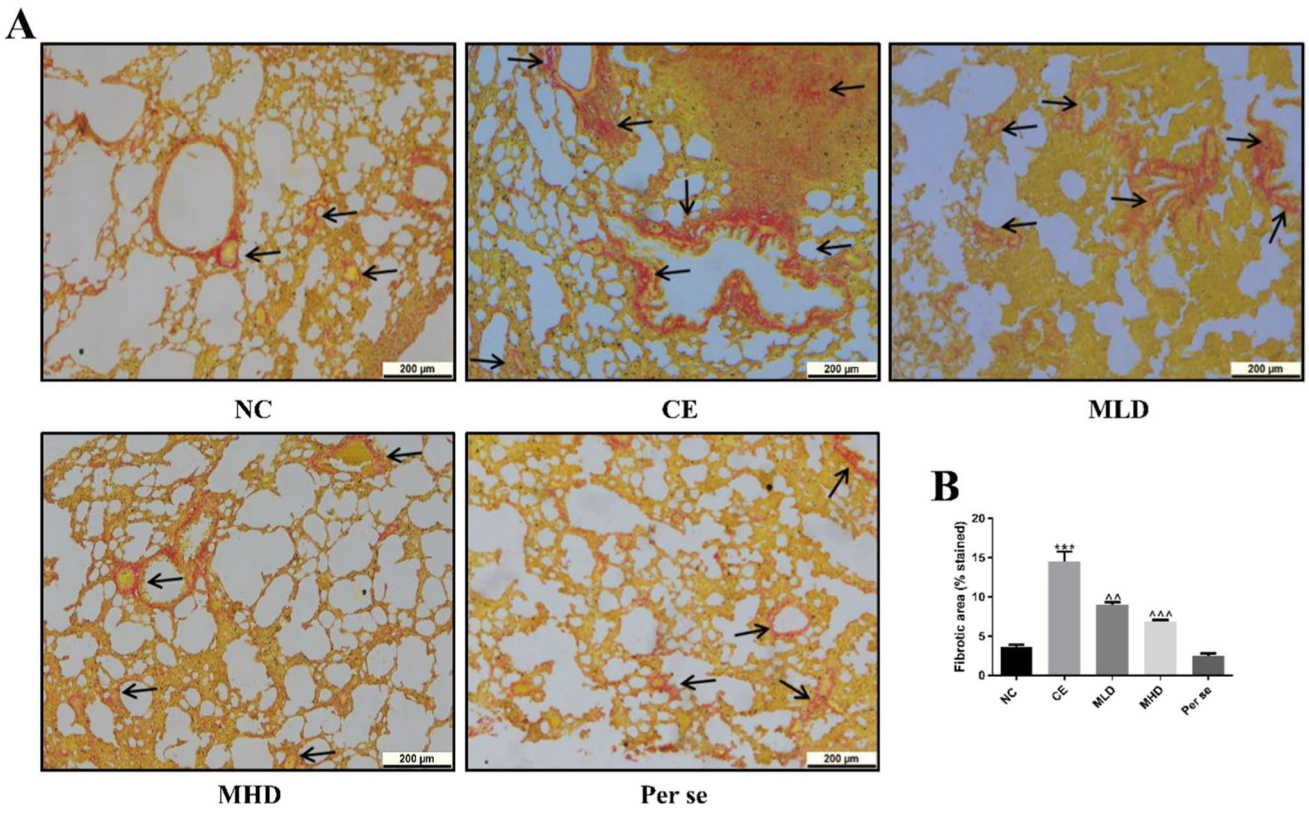

Fig. 7 Effect of melatonin on collagen deposition. **A** Representative images of lung sections stained with picrosirius red (red color (↑), collagen) as a marker of extracellular matrix deposition. **B** Quantitative analysis elucidating % area stained. Images were taken using light microscope (100 × magnification, scale bar = 200 µm). Results are expressed as mean ± S.E.M. ($n = 3$). ***$p < 0.001$ vs NC; ^^$p < 0.01$, ^^^$p < 0.001$ vs CE. NC, normal control; CE, copper exposed; MLD, copper + melatonin low dose (5 mg/kg); MHD, copper + melatonin high dose (10 mg/kg)

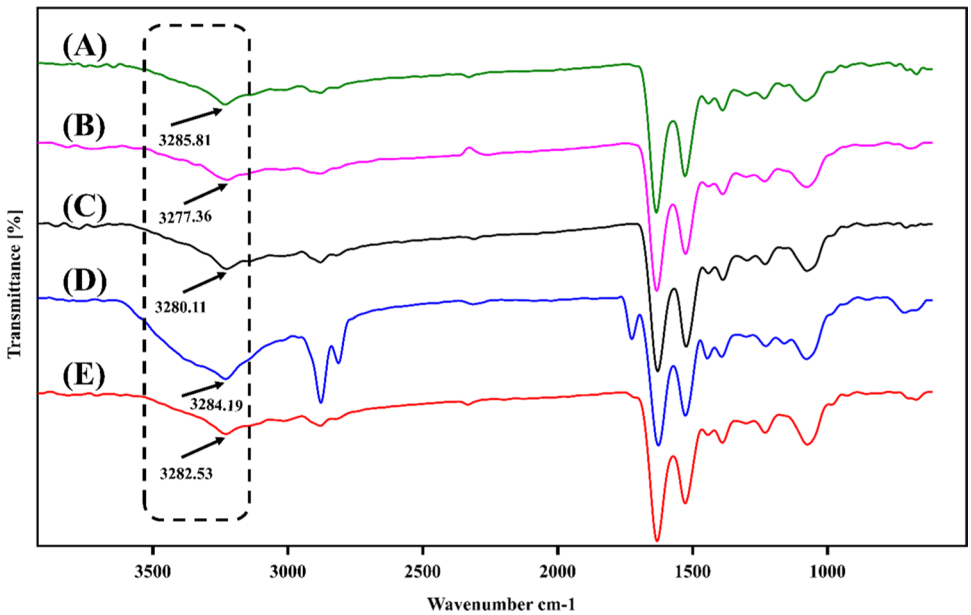

Fig. 8 Effect of melatonin on the hydroxyl functional group sift. **A** Normal control, **B** copper exposed, **C** copper + melatonin low dose (5 mg/kg), **D** copper + melatonin high (10 mg/kg) and **E** per se (melatonin alone 10 mg/kg). IR spectra of the lung tissue revealed that copper intoxication leads to changes in the frequency of -OH functional group sift. Treatment with melatonin normalized the frequency sift in animals intoxicated with copper. NC, normal control; CE, copper exposed; MLD, copper + melatonin low dose (5 mg/kg); MHD, copper + melatonin high dose (10 mg/kg)

Discussion

Copper is an essential trace element required for several basic activities in the human system, such as enzymatic transitions including biological and cellular processes (Kim et al. 2008; Lutsenko et al. 2019). Nonetheless, the dysregulated copper homeostasis and excessive copper deposition are associated with several pathological conditions (Li et al. 2018). Similarly, the increased release of effluents from the industries including copper is a global threat to human health, perturbating oxidative damages (Sebio et al. 2019). Our earlier findings have also revealed that copper intoxication is associated with the excess release of oxidative molecules in the liver tissue (Patwa and Flora 2020). Recently, several other researchers have also confirmed that copper accumulation is associated with tissue damage and organ fibrosis including lungs (Janssen et al. 2018; Padrilah et al. 2017). In addition, acute and chronic respiratory injury is linked with pneumonia, bacterial and viral infections such as severe acute respiratory syndrome (SARS-COVID-19), progressively a leading cause of mortality (Petrosillo et al. 2020; Spagnolo et al. 2020). Despite such challenges, no therapies are made available to treat the disease manifestations completely. However, the current available therapies provide symptomatic relief, necessitating the urgency to demand new platforms and treatments for managing pathological obliterations. Thus, pulmonary tissue being the most prominent and vital organ exposed to environmental metals and pollutants (Şimşek et al. 2021). In such a view, it is mandate to view its toxic effects and also to evaluate the effects of new and existing molecules for their efficacy profiles. One of the important things to mention is that the animals were exposed to copper through drinking water bottles mimicking the real situation, to study its consequent effect on the toxic outcomes. Complying with the existing literature and our preliminary observation, we have also considered the alarming situation of groundwater contamination with metals and its excessive usage in semi-conductor devices and utensils for various applications (Agrawal and Sahu 2010; Punia and Siva Siddaiah 2017). In addition, studies have shown that copper 20–200 mg/kg (orally) in rats have shown significant toxicity (Kumar et al. 2015; Patwa and Flora 2020). Central to this, the protective effects of melatonin in copper-induced lung injury remain unexplored. And the current study was designed to evaluate the protective effect of melatonin against copper-induced lung injury, with an emphasis on tissue oxidative stress and inflammation. Our results demonstrated that melatonin prevented the copper-induced lung injury. Administration of melatonin blocked the tissue oxidative stress, histological alterations and collagen deposition in copper-exposed animals. In addition, treatment with melatonin reduced the tissue copper levels and modulated serum ceruloplasmin levels induced by copper. Our results have further confirmed that oxidative stress primarily drives the tissue inflammation and injury. However, the observed beneficial effects might be mechanistically related to the ability of melatonin to prevent oxidative stress and tissue inflammation (Areti et al. 2017; Che et al. 2020; Negi et al. 2011). Grounded with the literature, we tried to integrate the usefulness of the current study with earlier findings to prevent the lung injury. Collectively, we envisage that such experimental findings will help in structuring the key solutions to the existing inefficiencies.

Diverse mechanisms and pathways have been elucidated for copper-induced lung toxicity. However, oxidative stress has been considered the major mechanism and prime culprit of metal-induced pathogenicity (Gaetke et al. 2014). Chronic copper administration results in the stimulation of antioxidative enzymes triggering various antioxidants (a Fenton type of reaction) (Aziz et al. 2020; Zeng et al. 2019). ROS and the end product of lipid peroxidation (MDA) are considered to be chief oxidative molecules contributing towards oxidative stress. MDA tends to cross-link with the lipids and proteins terminating them into toxic exudates (Liu et al. 2019). In addition, the redox imbalances are modulated by endogenous antioxidants like GSH and catalase (Zhao et al. 2019). These antioxidant enzymes help in the blockage of oxidative processes preferentially rendering them non-toxic (Somasundaram et al. 2019). In agreement, with the earlier published reports, we have also observed increased levels of oxidative radical's ROS, MDA and reduced antioxidant enzymes GSH and catalase in animals exposed to copper. Notably, treatment with melatonin modulated the levels of oxidative radicals and antioxidants enzymes. The significant reversal of hydroxyl radicals was strongly supported by the ability of melatonin to scavenge free radicals (Negi et al. 2011; Parmar et al. 2002), whereas the triggered oxidative responses are suggestive of the ability of metals to get deposited in the soft tissue which further grounds its phagocytosis potentiating apoptosis and tissue injury (Boveris et al. 2012; Patwa and Flora 2020). The excessive deposition of copper in the tissue reflects the induction of oxidative stress variables in the soft tissues (Gaetke and Chow 2003). Interestingly, earlier investigations with copper have shown that it impacts the liver, kidney and brain including lungs (Kumar et al. 2015; Zhang et al. 2021). And the degree of susceptibility to lung injury increases with time resulting in long-term respiratory peculiarities. Increased organ index after copper exposure represents the associated physiological abnormalities. However, no changes in the body weights were observed, which might due to its ability to promote growth. Reduction of body weight and increased organ index is reported after 100–300 mg/kg copper treatment in earlier studies

(Shahzad et al. 2012). Treatment with melatonin reduced the organ weight and organ index and the effects were more pronounced in the 10 mg/kg treated group. In addition, our findings have also indicated the increased levels of copper in the exposed animals. Parallel to this, studies have shown that MiADMSA and tetrathiomolybdate inhibit the accumulation of copper, by a mechanism forming stable complexes with the metal ions (Flora et al. 2008; Wei et al. 2011). The formed metal-ion complexes are taken up by the lysosomes and are subsequently released into the biliary ducts for elimination. Moreover, the deleterious effects of copper have been found to be diminished by the use of other metal chelators like glutathione (Fuentealba et al. 1993). Likewise, melatonin has also shown similar protective effects as glutathione and acts by forming complexes with copper in vitro (Limson et al. 1998). Paresh et al. suggested that melatonin strongly interacts with copper forming a new product. The formed product mechanistically chelates copper and reduces the deleterious effects (Parmar and Daya 2000; Parmar et al. 2002). Our results identified that melatonin administration decreases the deposition of copper in the exposed animals. Further, these observations are supported by the reduced levels of ceruloplasmin in the exposed animals. Ceruloplasmin a prominent marker was studied to evaluate the bound and unbound copper in the systemic circulation upon exposure. Several studies have reported the reduced ceruloplasmin in Wilson's disease, due to the mutation in ATP7A (Parmar et al. 2002; Patil et al. 2013; Roberts and Schilsky 2008). Nevertheless, studies have also evaluated increased levels of ceruloplasmin activity in copper-exposed rats (Park et al. 2014). These can be further connected to the availability of different experimental models to study the disease physiology. Recently, metal chelators like D-penicillamine and trientine are used to reduce the symptoms of Wilson's disease via modulating ceruloplasmin activity (Bandmann et al. 2015). Likewise, administration of melatonin modulated the low ceruloplasmin, where it was found to reduce the clinical presentation of Wilson's disease in several experimental models (Adamczyk-Sowa et al. 2016; Sharma et al. 2019; Wang et al. 2019).

In parallel, previous investigators have identified that inflammation plays a key pathological role in the progression of several diseases (Chen et al. 2018; Wynn 2011). In addition, the role of macrophages has been widely studied to understand the pathogenesis of lung injury due to its imperative role in the activation of inflammasome and inflammation. However, the role of macrophages has not been studied in the current study but still, evidences have documented its definite role in triggering tissue damage regulated by inflammation (Wu and Lu 2020). Moreover, researchers have also shown that ROS-mediated oxidative stress stimulates the inflammatory response. However, inflammation acts as an imperative defense system protecting cellular and biological properties of the cell and tissue via acting on external stimuli. And the nature of inflammatory response induced by copper is dependent on the recruitment of inflammatory cells like (neutrophils, alveolar macrophages and lymphocytes) and release of cytokines and chemokines (Lominadze et al. 2004). Remarkably, copper-based complexes are reported to show anti-inflammatory properties along with other non-steroidal anti-inflammatory drugs both in vivo and in vitro (Hussain et al. 2019). Conventionally, copper was also used to treat inflammation-regulated autoimmune diseases like rheumatoid arthritis in reversing the cartilage injury advanced due to arthritic inflammation (Walker and Keats 1976). However, the mechanism behind the observed protective effects remains unexplained. When the levels of copper exceed normal human tolerated levels, it results in the activation of several signalling cascades related to inflammation such as NF-κB and MAPKs (Yang et al. 2020). The histopathological investigations suggested the increased thickening of alveolar walls and reduced airway spaces in animals challenged with copper. Treatment with melatonin to an extent reduced the tissue abnormalities. Similar kinds of observations were reported earlier by several researchers that aggravated tissue injury induces lung dysfunction (Ali et al. 2021b; Pooladanda et al. 2019; Zhou et al. 2018). In addition, increased inflammatory cell infiltration in animals challenged with copper correlated well with other published reports which showed increased inflammatory cell infiltration in diseased tissue (Lai et al. 2018; Pooladanda et al. 2019). Earlier findings have also shown that melatonin treatment reduced LPS-induced inflammatory responses in stimulated bovine epithelial cells (Yu et al. 2017). Negi et al. demonstrated that melatonin treatment reduced streptozotocin-induced diabetic neuropathy via reducing NF-κB and Nrf2 pathways (Negi et al. 2011). In addition, our previous investigations also proved that the use of antioxidant and anti-inflammatory agents like MiADMSA reduced tissue inflammation and injury in animals challenged with copper (Patwa and Flora 2020).

To further understand the link between inflammation and tissue injury, we have analysed lung sections for collagen deposition, which gets accumulated in the tissue in response to aggravated inflammatory responses. The induction of collagen deposition is strongly correlated with lung tissue fibrosis and several other diseases leading towards the irreversible scarring of tissue and pulmonary insufficiencies (Kim et al. 2011; Lai et al. 2018). We also noticed that the collagen deposition was increased in animals exposed to copper indicative of copper-induced lung toxicity. Our observations were similar with the earlier published evidences which indicated that copper stimulates endothelial cells inducing collagen deposition (Gérard et al. 2010). In agreement, previous reports indicated decreased collagen deposition and pulmonary tissue injury upon melatonin

treatment further supporting our findings (Arslan et al. 2002; Shin et al. 2017). Inline, the protective effects of melatonin by which it attenuates pulmonary fibrosis remain unclear. Nonetheless, based on the evidences, it can be anticipated that melatonin blocks early-stage lung inflammation which might be involved in its anti-fibrotic effects.

The effect of melatonin was further investigated by performing spectral analysis. As exposure of metals gets accumulated in soft tissues including lungs, resulting in sift in the hydroxy functional groups towards lower frequency wavelength (Boveris et al. 2012; Patwa and Flora 2020). In acceptance to this, we performed IR spectra to determine characteristic sift in the functional groups. The representative alcoholic/phenolic -OH group peaks were observed in the IR spectra recorded at 3285–3794 cm^{-1} (Haris and Chapman 1992). And we investigated that melatonin treatment normalized the shift in -OH functional group towards higher frequency region altered by copper. However, evidences supporting the effects of melatonin in reversing the functional group changes are not available. Interestingly, our previous findings suggested that the ability of metal chelators to bind with the metals and biomolecules might be involved in the observed effects (Patwa and Flora 2020). Collectively, the results on oxidative stress and tissue inflammation ensuing lung injury indicated that melatonin significantly improved tissue inflammation and airway injury in the exposed animals. Not surprisingly, the present study is also meant with certain limitations like lack of perfect preclinical models, and the preliminary observations of the current study are based on the ability of melatonin to ameliorate oxidative stress and tissue inflammation. Another thing to note is that the animals were treated with melatonin after the chronic exposure of copper, whereas studies evaluating the effects upon concurrent treatment might yield different results. Lastly, the disease advances via diverse pathways. Therefore, studying detailed molecular mechanism and its subsequent fate on PK-PD parameters in future could be a more worthwhile approach to combat the lung injury and other related pathologies.

Conclusion

In conclusion, the present study provides some new and interesting observations regarding the therapeutic efficacy of melatonin against copper intoxication in rats. The current findings suggested that the beneficial effects of melatonin against copper-induced pulmonary injury are mediated principally through the reduction in oxidative stress, inflammation and histopathological changes. And the findings suggest that melatonin could be considered a potential therapeutic candidate for treating inflammatory complications intricating disease progression and tissue fibrosis. However, further studies are required to validate the beneficial effects of melatonin in treating pulmonary complications.

Author contribution S.G. and S.A.A. performed all the experiments, analysed the data, prepared and revised the manuscript. P.S. prepared the first draft of the manuscript. J.P., S.J.S. and AKD designed the study. A.K.D. supervised the work, interpreted the results, prepared the manuscript draft and corrected the final manuscript.

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Data availability The study data are available on request to corresponding author.

Declarations

Ethics approval Experimental protocol approved by Institutional Animal Ethics Committee (IAEC), NIPER-Raebareli.

Consent to participate Not applicable.

Consent for publication All authors agreed for submission in Environmental Science and Pollution Research.

Competing interests The authors declare no competing interests.

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