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Circulatory heavy metals (cadmium, lead, mercury, and chromium) inversely correlate with plasma GST activity and GSH level in COPD patients and impair NOX4/Nrf2/GCLC/GST signaling pathway in cultured monocytes

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ABSTRACT

This study aims to examine the hypothesis that circulatory heavy metals may be associated with lung function decline and lower plasma GST activity and GSH level in COPD patients via activating monocytes mediated by impairing the NOX4/Nrf2/GCLC/GST signaling pathway. Results showed that the blood levels of heavy metals (cadmium, lead, mercury, and chromium) were significantly higher in COPD patients of coal mine site compared to the healthy controls. The levels of heavy metals in COPD patients were significantly and negatively correlated with lung function, GST activity, and GSH level. Using flowcytometry, fluorescence spectroscopy, and immunoblotting studies we have further demonstrated that treatment with individual heavy metals dose-dependently increased the NOX4 protein expression, intracellular ROS production, and decreased the Nrf2, GCLC, and GST protein expression, GST activity, and GSH level in THP-1 monocytes. None of the treatment caused any change in cell viability compared to control. In conclusion, this study suggests that circulatory heavy metals in COPD patients of coal mine site weakened the lung function, decreased the plasma GST activity and GSH level via impairing the NOX4/Nrf2/GCLC/GST signaling pathway in monocytes, which may cause monocyte activation and initiate the COPD pathophysiology.

1. Introduction

Occupational exposure to heavy metals, toxic gases, dust, or fumes are associated with the development of chronic obstructive pulmonary disease (COPD), a major cause of morbidity and mortality worldwide (Boschetto et al., 2006; Kraim-Leleu et al., 2016; Rokadia and Agarwal, 2013). Several epidemiological studies have shown the association of heavy metal exposure with lower pulmonary function, a hallmark for developing COPD (Heo et al., 2017; Little et al., 2017; Oh et al., 2014). There has been an increased exposure of heavy metals to the environment due to various anthropogenic factors, such as coal mining, smelting, and industrial effluents. Upon metabolism within the body, heavy metals, particularly lead, mercury, and cadmium, produce a large amount of reactive oxygen species (ROS) and cause oxidative impairment in various organs (Agrawal et al., 2014; Almeida Lopes et al., 2017; Patra et al., 2011; Rehman et al., 2018).

Glutathione-s-transferase (GST) is a super family of enzymes comprising alpha, mu, pi, theta, kappa, zeta, sigma, omega, and delta sub unitsthat are involved in the detoxification mechanism via conversion of many endogenous and exogenous electrophilic compounds to less reactive metabolites (Nebert and Vasiliou, 2004). A series of earlier studies including our previous report demonstrated that genetic polymorphism of GST (GSTM1, GSTT1, and GSTP1) is a critical risk factor for the development of COPD among various populations (Dey et al., 2014; Ishii et al., 1999; Lakhdar et al., 2011; Rodriguez et al., 2005; Shukla et al., 2011). The total antioxidant capacity was reported to be

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lower in COPD subjects with deletion of GSTM1 and GSTT1 genes compared to healthy control (Cao et al., 2017). It was reported that the erythrocyte GST enzyme activity was positively correlated with lung function (FEV1, % predicted) in COPD patients (Mohammed et al., 2017). Furthermore, glutathione (GSH), the tri-peptide antioxidant primarily responsible for preventing cellular oxidative damage, isalso reported to be associated with the severity of COPD (Dey et al., 2016; Elmasry et al., 2015). All these studies demonstrated the association of GST polymorphism/activity and GSH levels with pathogenesis of COPD among various populations. However, there is no study in the literature that has examined the responses of occupational heavy metal exposure on the regulation of plasma GST activity and GSH level in relation to the development of COPD. Moreover, the molecular mechanism underlying the effect of heavy metal on GST activity and GSH level remains unclear.

The nuclear factor erythroid 2 (NFE2)-related factor 2 (Nrf2) is playing an important role in regulating the transcription of antioxidant enzymes (Loboda et al., 2016). Besides,the NADPH oxidase (NOX) system comprising NOX1, NOX2, NOX4, and NOX5 isoforms is being recognised as a primary source of ROS production (Troiano et al., 2016). Liu et al. reported that the abundance of NOX4 protein in the airway smooth muscle cells of COPD patients was increased with disease severity and also negatively associated with the pulmonary functions (Liu et al., 2016). Using Nrf2 knockout mice model, Ishii et al. demonstrated the protective role of Nrf2 against lung inflammation via the activation of antiprotease and antioxidants in alveolar macrophages (Ishii et al., 2005). Circulatory monocytes play an important role in the pathogenesis of lung function decline in COPD via migrating into lungs followed by infiltrating into alveolar space to form alveolar macrophages, which mediate the inflammatory processes (Aldonyte et al., 2003; Jiang et al., 2017). The current study for the first time examined the relationship between circulatory heavy metals (cadmium, lead, mercury, and chromium) concentrations, GST activity, GSH level, and lung function in COPD patients of coal mine site and further dissected the direct effect of individual heavy metal on intracellular ROS production, GST activity, GSH level, and NOX4/Nrf2/GCLC/GST signaling pathway in THP-1 monocytic cells.

2. Material and methods

2.1. Chemicals

All chemicals are purchased from Sigma (St. Louis, USA) unless otherwise mentioned. Human specific antibodies were purchased from Abcam, Inc. (Cambridge, MA).

2.2. Study enrollment and blood sample collection from COPD patients and control subjects

The study was conducted at CSIR-North East Institute of Science and Technology, Jorhat, Assam after ethical clearance from the Institutional Ethics Committee (IEC), NEIST, Jorhat. Informed written consent was obtained from all COPD patients and control subjects according to the protocol approved by the Indian Council of Medical Research (ICMR). To observe a significance changes from baseline, the required sample size was calculated at 5% significance level and 95% power. The final study population comprised of 93 COPD patients (non smoker) (58 male and 35 female; mean age 54 ± 1.09 years), who were examined by the doctors of Clinical Centre, NEIST, Jorhat and Local Public Health Centre of Jaintia hill and recommended for blood sample collection after conducting spirometry during the health camp. Age and gender-matched 85 non-smoker,control subjects (55 male and 30 female; mean age 51 ± 0.96) were enrolled in the study from Jorhat, Assam, where no such coal mine area exists. The blood samples were collected in EDTA tubes and the clear plasma were separated via centrifugation at 3000 rpm for 15 min.

Table 1
Total population, age, M/F ratio, BMI, FEV1/FVC (% predicted), GST activity, and GSH levels in patients with COPD and healthy controls. Values are mean ± SE. "*" denotes the significant difference from healthy control ($p^* < 0.05$).

Parameter	COPD	Control
Total population (n)	93	85
Age (years)	54 ± 1.09	51 ± 0.96
Male/female	58/35	55/30
BMI (Kg/m^2)	16.30 ± 0.85*	21.45 ± 0.56
FEV1/FVC (% predicted)	52.40 ± 2.49*	80.40 ± 1.89
Plasma GST activity (μmol/min/mL)	2.204 ± 0.11*	8.401 ± 0.14
Plasma GSH (μmol/mL)	4.705 ± 0.132*	8.50 ± 0.21

Table 2
Measurement of the concentration of heavy metals in blood of COPD patients and healthy controls through Atomic absorption spectroscopy analysis. Values are expressed as mean ± SE. "*" denotes the significant difference from healthy control ($p^* < 0.05$)

Heavy metal	Concentration (μg/mL)	
	COPD	Healthy
Cadmium	0.0207 ± 0.003*	0.0025 ± 0.001
Lead	0.0293 ± 0.002*	0.0039 ± 0.001
Mercury	0.3104 ± 0.022*	0.0418 ± 0.003
Chromium	0.0086 ± 0.001*	0.0023 ± 0.001

2.3. Estimation of blood level of heavy metals among COPD patients and control subjects

The levels of blood heavy metals such as cadmium (Cd), Lead (Pb), mercury (Hg), and chromium (Cr) in COPD patients of coal mine site and control subjects were measured using Atomic Absorption Spectroscopy (AAS) (Perkin Elmer, A Analyst-700) as per the method described by Clinton (Clinton, 1977). Briefly, the blood samples were digested with perchloric acid and nitric acid mixture at 210 °C for 60 min. The samples were then allowed to cool, and hydrochloric acid is added in sufficient amount. A series of standards of all the elements were prepared accordingly, and the absorbance level was compared with the experimental samples. Three replicates were measured for each sample.

2.4. Cell culture studies

2.4.1. Human THP-1 monocytic cell line

The human THP-1 monocytic cell line was obtained from the National Centre for Cell Sciences (Pune, India). Cells were maintained at 37 °C in RPMI 1640 medium containing 5.5 mM glucose, 10% (v/v) FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 12 mM sodium bi-carbonate, and 25 mM HEPES in a humidified atmosphere containing 5% (v/v) CO_2.

2.4.2. Treatment of cells with heavy metals

The heavy metals selected for the in vitro study were cadmium (Cd), lead (Pb), mercury (Hg), and chromium (Cr), and the chemical sources for these heavy metals were cadmium chloride ($CdCl_2$), lead chloride ($PbCl_2$), mercuric chloride ($HgCl_2$), and chromium chloride ($CrCl_3$), respectively, where water was used as the solvent. The concentrations of heavy metals chosen for the cell culture study were based upon the Atomic Absorption Spectroscopy analyses of the blood levels of heavy metals among COPD patients and control subjects, and the selected doses were as follows: Cd (0.002, 0.004, 0.008, 0.016 and 0.032 μg/mL), Pb (0.003, 0.006, 0.012, 0.024 and 0.048 μg/mL), Hg (0.004, 0.008, 0.016, 0.032 and 0.064 μg/mL), and Cr (0.001, 0.002, 0.004, 0.008 and 0.016 μg/mL). THP-1 monocyte cells were treated with all

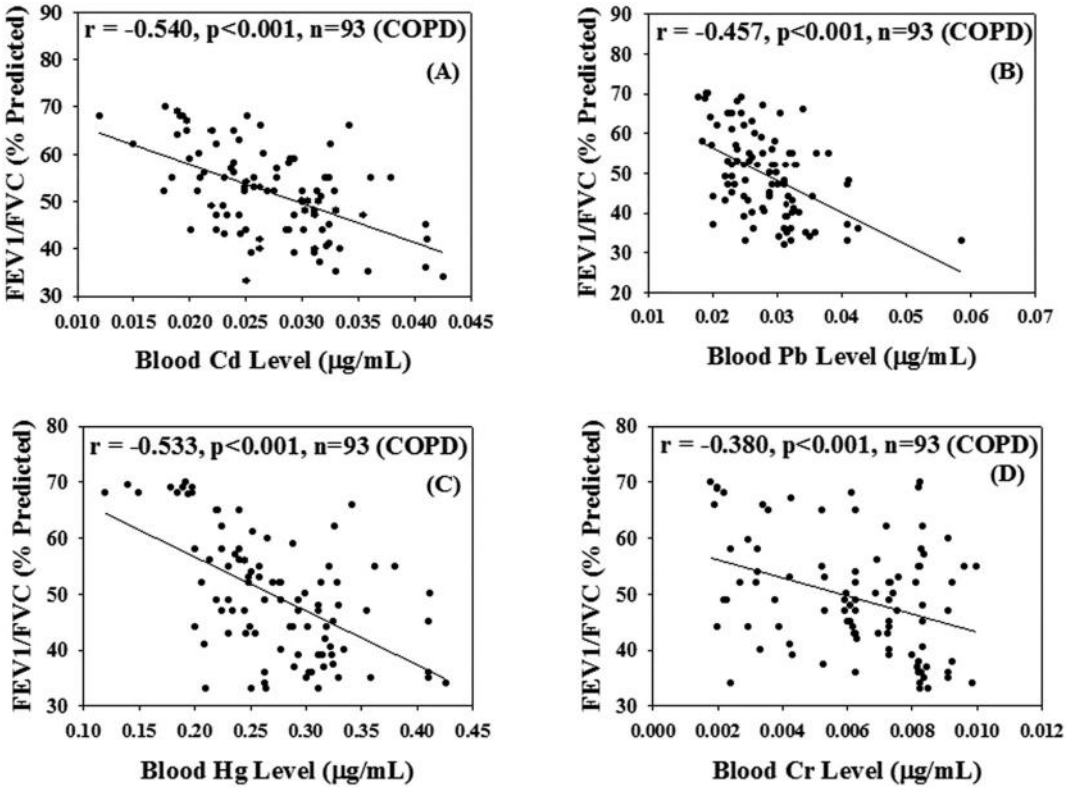

Fig. 1. The relationship between blood levels of heavy metals (Cd, Pb, Hg, and Cr) and lung function (FEV1/FVC, % predicted) among COPD patients (A-D).

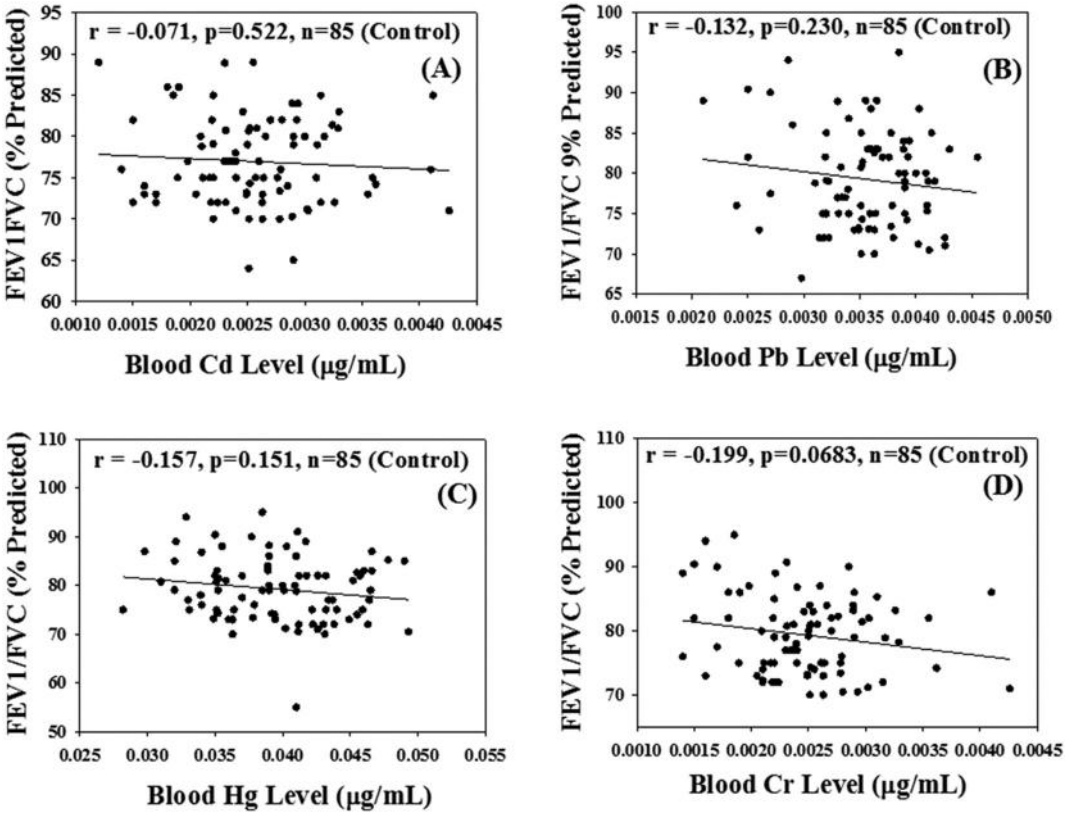

Fig. 2. The relationship between blood levels of heavy metals (Cd, Pb, Hg, and Cr) and lung function (FEV1/FVC, % predicted) among control subjects (A-D).

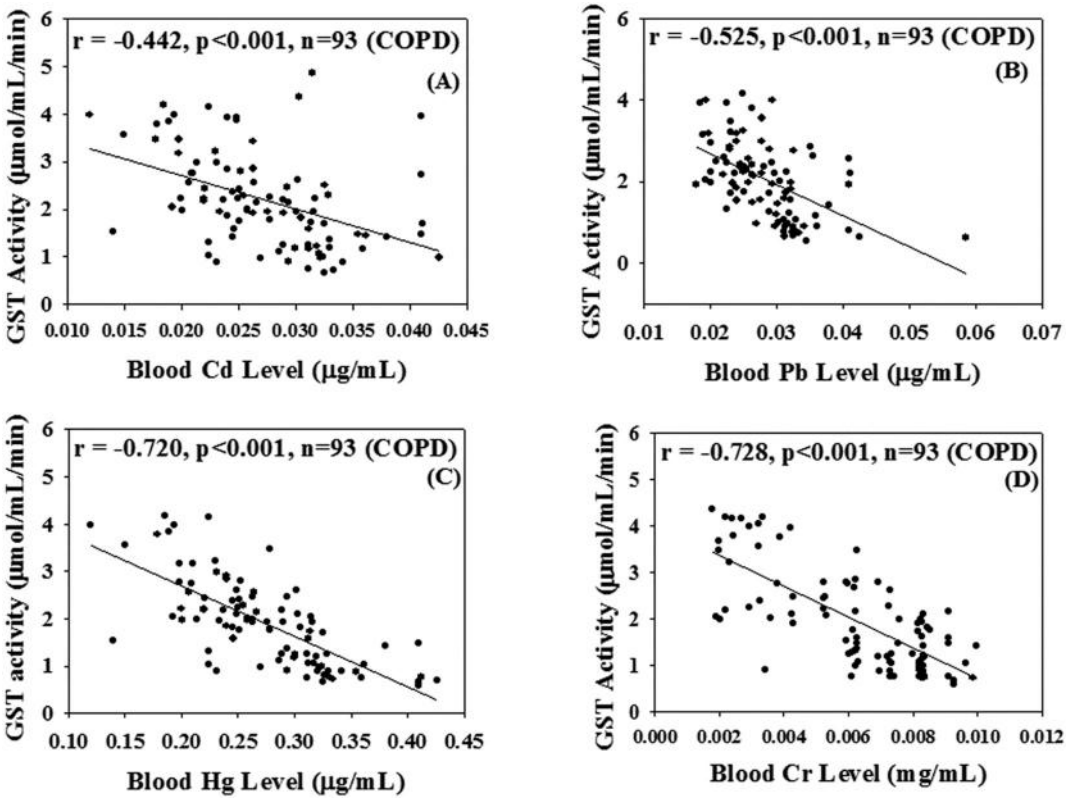

Fig. 3. The relationship between blood levels of heavy metals (Cd, Pb, Hg, and Cr) and plasma GST activity among COPD patients (A-D).

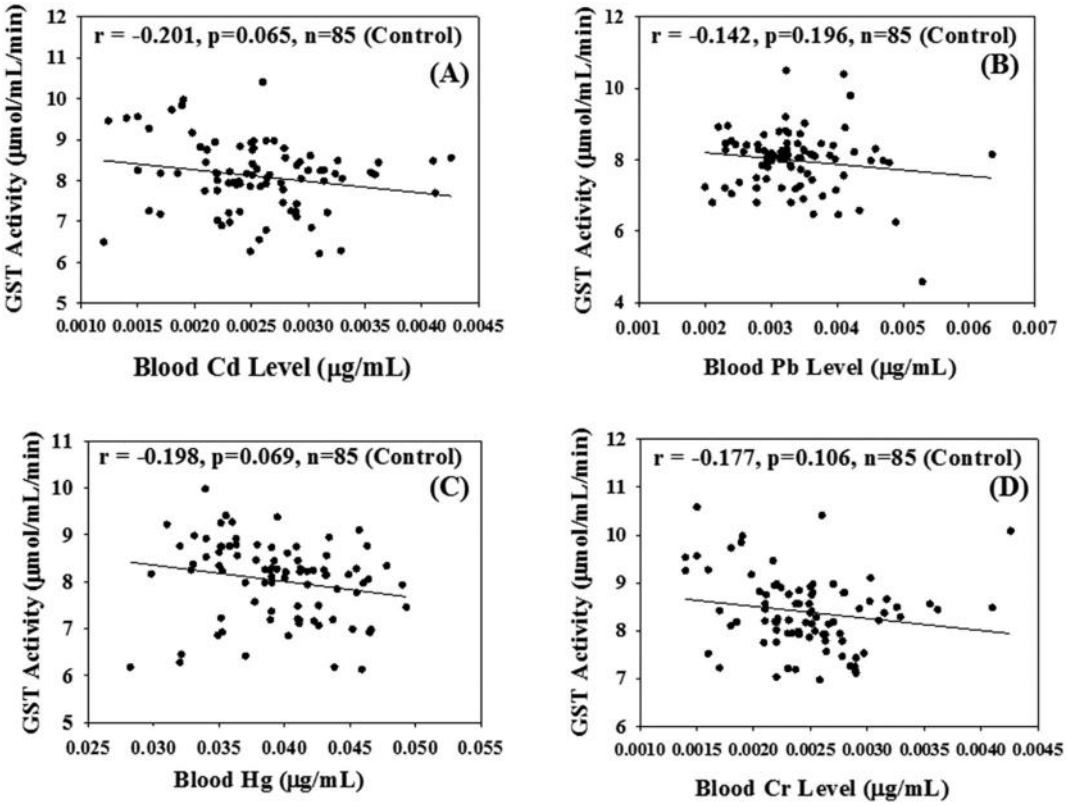

Fig. 4. The relationship between blood levels of heavy metals (Cd, Pb, Hg, and Cr) and plasma GST activity among control subjects (A-D).

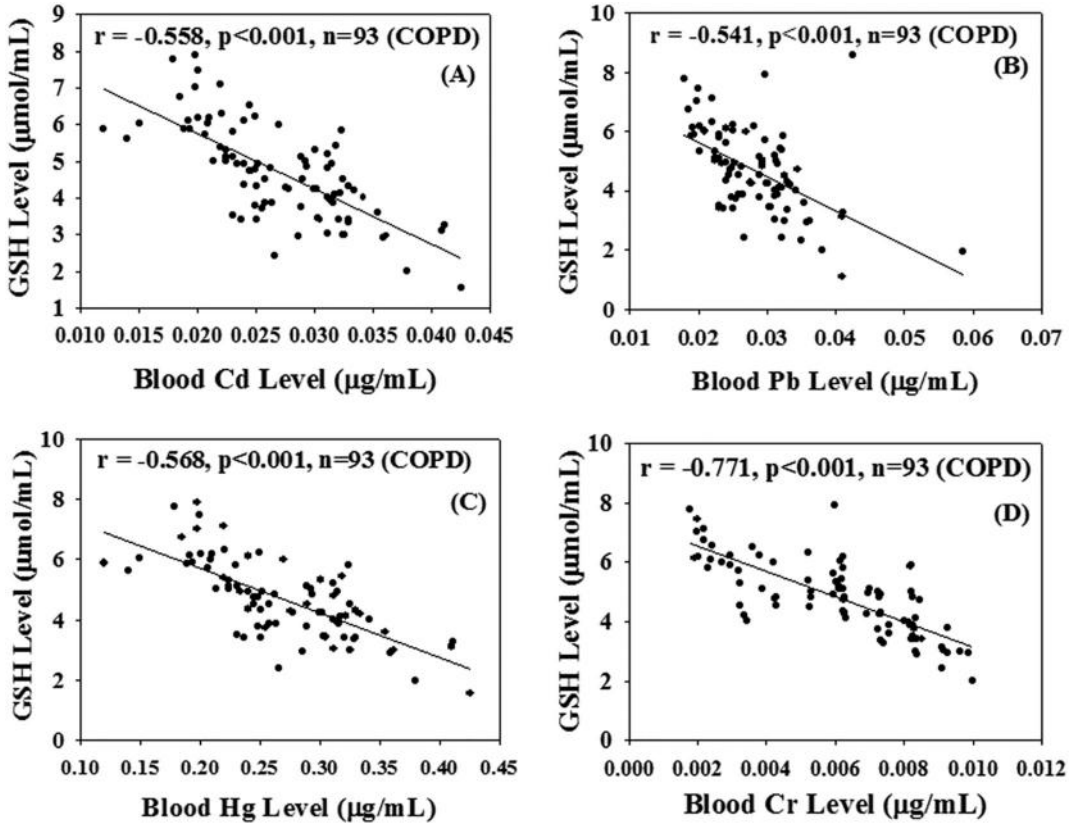

Fig. 5. The relationship between blood levels of heavy metals (Cd, Pb, Hg, and Cr) and plasma GSH levels among COPD patients (A-D).

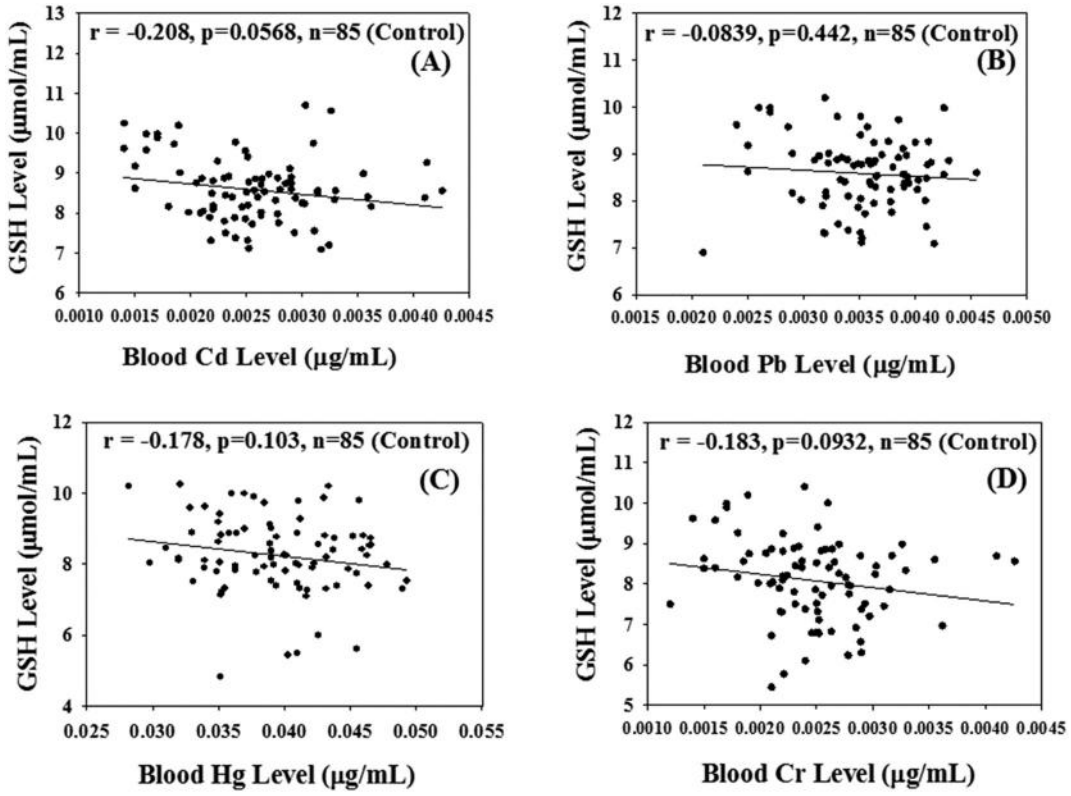

Fig. 6. The relationship between blood levels of heavy metals (Cd, Pb, Hg, and Cr) and plasma GSH levels among control subjects (A-D).

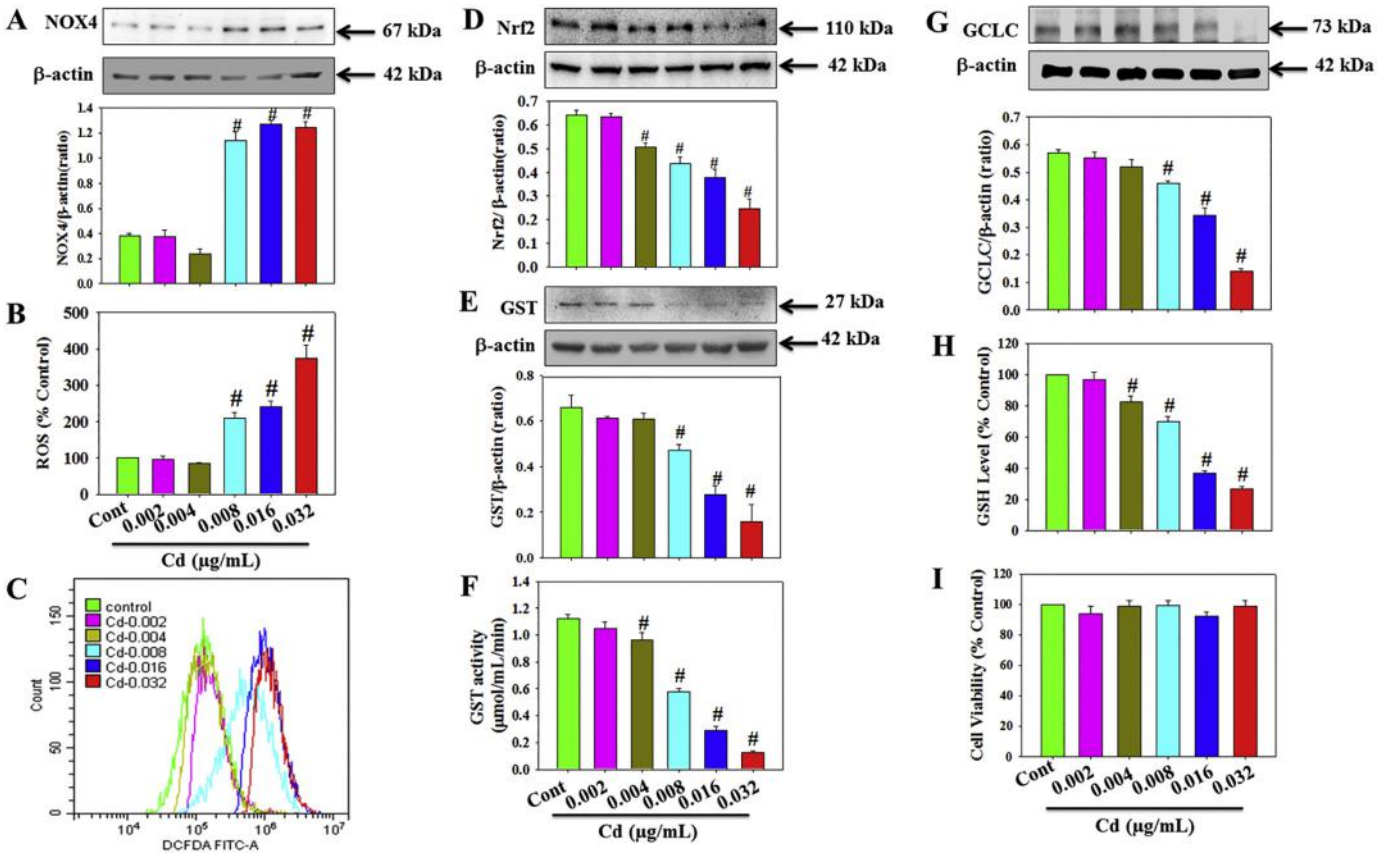

Fig. 7. Effect of Cd on NOX4 protein expression (A), intracellular ROS level using fluorescence spectroscopy (B) and Flowcytometry (C), Nrf2 protein expression (D), GST protein expression (E), GST activity (F), GCLC protein expression (G), GSH level (H), and Cell viability (I) in THP-1 monocyte cells. Cells were pre-treated with increasing doses (0.002–0.032 μg/mL) of $CdCl_2$ (as a source of cadmium) for 24 h at 37 °C. Values are mean ± standard error. "#" denotes the significance difference from control group ($p\# < 0.05$).

these heavy metals for 24 h at 37 °C. After treatment, cells were washed in phosphate buffer saline (PBS) and lysed in radio immunoprecipitationassay (RIPA) buffer containing 50 mM Tris in pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% SDS supplemented with protease and phosphatase inhibitors (1 mMPMSF, 5 mg per mL leupeptin, 2 mg/mL, aprotinin, 1 mMEDTA, 10 mMNaF, and 1 mM Na_3VO_4). Lysates were cleared using centrifugation, and total protein concentrations were determined using the BCA assay as per the manufacturer'sprotocol (Pierce/Thermo Scientific, Rockford, IL).

2.4.3. Cell viability assay

Cell viability test was performed using the Alamar Blue reduction bioassay. This method is based upon Alamar Blue dye reduction by live cells. Briefly, the monocyte cells were seeded at a concentration of 1 million cells/well in a 96 well plate. The cells were then treated with respective concentration of the heavy metals considered for the study. After the treatment with heavy metals for 24 h, the Alamar Blue dye at a concentration of 0.4% was added to the plate. The plate was then incubated at 37 °C in a CO_2 incubator for 4 h. The absorbance was measured at 570 nm and 600 nm. The result was expressed as percentage over control (Manna and Jain, 2014).

2.5. Detection of intracellular ROS levels

Intracellular reactive oxygen species (ROS) levels were determined using the fluorescent dye DCFDA (2′,7′-dichlorofluoresceindiacetate). After treatment with heavy metals (Cd, Pb, Hg, and Cr), cells were washed once with PBS, then loaded with 1 μM DCFDA. The cells were incubated at 37 °C for 30 min in the dark, subsequently washed with PBS, and centrifuged at 10000 *g* for 10 min. After washing, the intracellular ROS level of the cells was measured under Fluorescence Spectrophotometer (Fluorolog, Horiba Scientific) at excitation and emission wavelengths of 466 and 530 nm, respectively. The fluorescence intensity of 10,000 cells for DCFDA was also assessed by employing a Flow cytometer (CytoFLEX, Beckman Coulter).

2.6. Estimation of Glutathione S-transferase (GST) enzyme activity in human plasma and THP-1 monocytes

GST activity was measured in plasma sample of COPD and control subjects as well as cell lysates of cells treated with heavy metals as per the method described by Habig et al. (Habig et al., 1974). The reaction mixture contained a suitable amount of the enzyme, KH_2PO_4 buffer (pH 7.4), CDNB (1 mM), and GSH (6 mM). The reaction was carried out at 37 °C and monitored spectrophotometrically by the increase in absorbance of the conjugation of GSH with CDNB at 340 nm for 3 min. One unit of GST activity is defined as 1 μmol of product formation per minute per mL of sample.

2.7. Estimation of Glutathione (GSH) level in human plasma and THP-1 monocytes

GSH level was measured using the method explained by Dey et al. in blood samples of COPD and control subjects and heavy metals treated cell lysates (Dey et al., 2016). The precipitating solution containing 1 mM EDTA and 100 g/L TCA was added to 500 μLplasma to precipitate the protein contents and centrifuged at 15,000 rpm for 10 min. After centrifugation DTNB solution was added to the supernatant and measured the absorbance at 412 nm using microplate reader. Suitable amount of 0.3 M Na_2HPO_4 (disodium phosphate) and DTNB solution

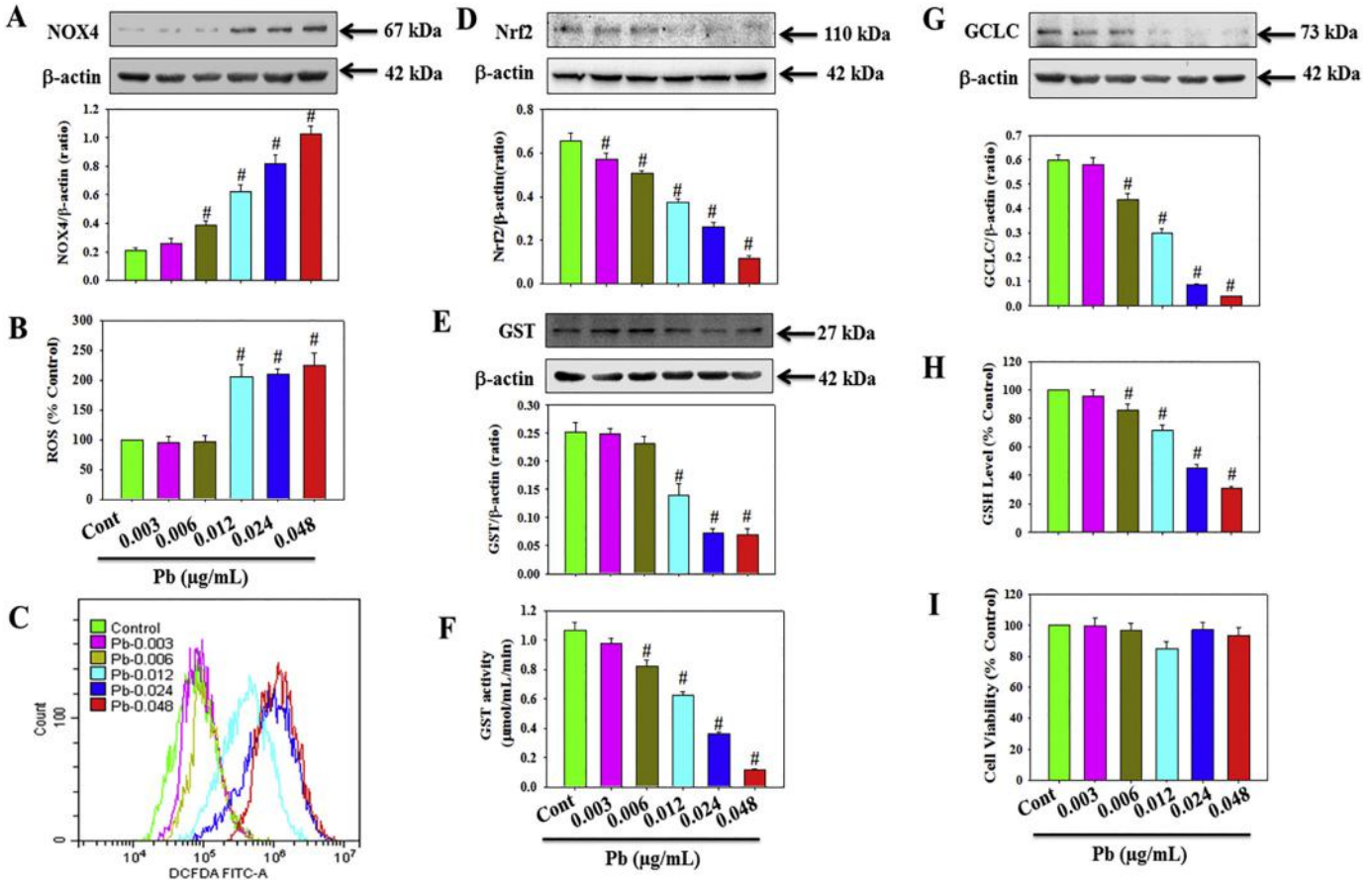

Fig. 8. Effect of Pb on NOX4 protein expression (A), intracellular ROS level using fluorescence spectroscopy (B) and Flowcytometry (C), Nrf2 protein expression (D), GST protein expression (E), GST activity (F), GCLC protein expression (G), GSH level (H), and Cell viability (I) in THP-1 monocyte cells. Cells were pre-treated with increasing doses (0.003–0.048 μg/mL) of $PbCl_2$ (as a source of lead) for 24 h at 37 °C. Values are mean ± standard error. "#" denotes the significance difference from control group ($p\# < 0.05$).

were added to 10 μL of cell lysates, and the GSH level measured spectrophotometrically at 412 nm.

2.8. Immunoblotting

Protein samples (~20 μg each) from whole cells were subjected to SDS PAGE gel electrophoresis (8–10%) in 1× running buffer (25 mM Tris, 190 mM Glycine, 0.1% SDS, and distilled water), initially at 70 V approximately for 1 h and then at 100 V until completion. The gel was then transferred to a nitrocellulose membrane using 1× transfer buffer (25 mM Tris, 190 mM Glycine, 20% Methanol, and distilled water) at 24 V for 1 h. Membranes were blocked at room temperature for 2 h in blocking buffer containing 1% BSA to prevent nonspecific binding and then incubated with primary antibodies: anti-NOX4 (1:1000) (# ab133303), anti-Nrf2 (1:1000) (# ab137550), anti-GCLC (1: 25) (# ab53179), and anti-GST (1:1000) (# ab108524) overnight at 4 °C. The membranes were washed in TBS-T (50 μmol L^{-1} Tris HCl, pH 7.6, 150 μmol L^{-1} NaCl, and 0.1% Tween 20) for 30 min (three times) and incubated with the appropriate HRP conjugated secondary antibody (1:4000) for 2 h at room temperature. Images were developed using the ultrasensitive ECL substrate (Bio-Rad). The intensity of each immunoblotting band was measured using the histogram tool of Adobe Photoshop.

2.9. Statistical analysis

Data were analyzed statistically using one way analysis of variance (ANOVA) with Sigma Stat statistical software (JandelScientific, San Rafael, CA). All groups were compared using the Student–Newman–Keulspost hoc method. Student *t*-test was used to compare blood levels of heavy metal in COPD and control group. Pearson product moment correlation analysis was used to determine the relationship of circulatory heavy metals with lung function, plasma GST activity, and GSH level among both control and COPD individuals. A p value of < 0.05 was considered significant for a statistical test. The data were represented as mean ± SE.

3. Results

3.1. Baseline characteristics and blood levels of heavy metals among the studied population

Table 1 represents a significant ($p < .05$) decrease in body mass index (BMI) (16.30 ± 0.85 vs. 21.45 ± 0.56 Kg/m^2), lung function (FEV_1/FVC ratio) (52.40 ± 2.49 vs. 80.40 ± 1.89% predicted), and plasma GST activity (2.204 ± 0.11 vs 8.401 ± 0.14 μmol/min/mL) and GSH level (4.705 ± 0.132 vs 8.50 ± 0.21 μmol/mL) among COPD patients of coal mine site compared to healthy controls. However, the mean age differences between the COPD and control group were statistically insignificant. Atomic Absorption Spectroscopy analyses (Table 2) demonstrated a significant ($p < .05$) elevation in levels of plasma heavy metals, namely Cd (0.0207 ± 0.003 vs. 0.0025 ± 0.001 μg/mL), Pb (0.0293 ± 0.002 vs. 0.0039 ± 0.001 μg/mL), Hg (0.3104 ± 0.022 vs. 0.0418 ± 0.003 μg/mL), and Cr (0.0086 ± 0.001 vs. 0.0023 ± 0.001 μg/mL) in COPD patients compared to control group.

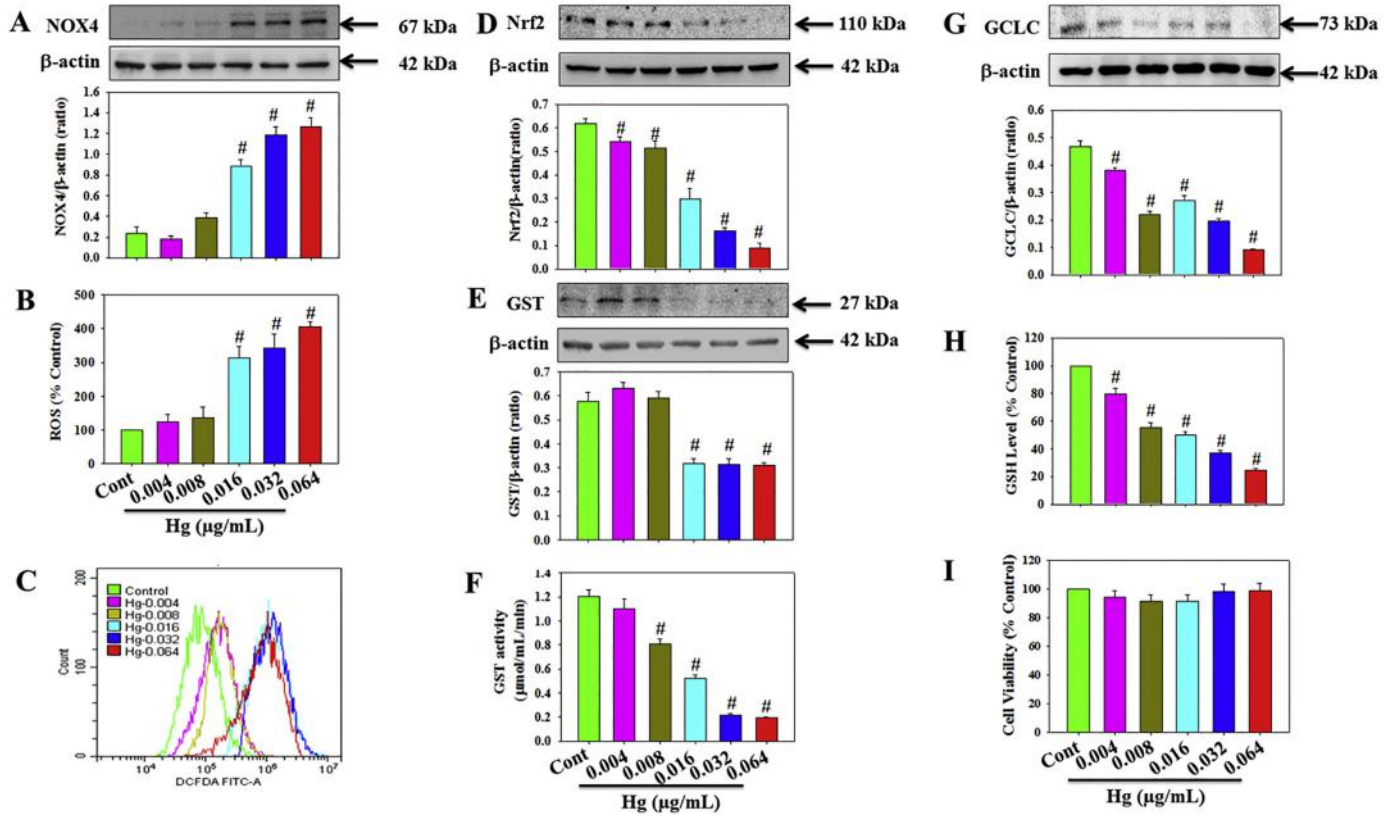

Fig. 9. Effect of Hg on NOX4 protein expression (A), intracellular ROS level using fluorescence spectroscopy (B) and Flowcytometry (C), Nrf2 protein expression (D), GST protein expression (E), GST activity (F), GCLC protein expression (G), GSH level (H), and Cell viability (I) in THP-1 monocyte cells. Cells were pre-treated with increasing doses (0.004–0.064 μg/mL) of $HgCl_2$ (as a source of mercury) for 24 h at 37 °C. Values are mean ± standard error. "#" denotes the significance difference from control group ($p\# < 0.05$).

3.2. Relationship of blood heavy metals with lung function, GST activity, and GSH level among COPD patients and control subjects

Fig. 1 illustrates that there was a statistically significant ($p < .001$) inverse correlation between the circulating levels of heavy metals (Cd, Pb, Hg, and Cr) and lung function (FEV1/FVC %, predicted) among COPD patients. Similarly, the levels of blood heavy metals (Cd, Pb, Hg, and Cr) were also significantly ($p < .001$) and negatively correlated with plasma GST activity and GSH level among COPD patients (Figs. 3 and 5). However, a weak and statistically non-significant ($p > .05$) inverse correlations were observed between the levels of blood heavy metals (Cd, Pb, Hg, and Cr) with lung function (Fig. 2), GST activity (Fig. 4), and GSH level (Fig. 6) among the control subjects. These results indicate that exposure to heavy metals may have a detrimental effect in lowering lung function, GST activity, and GSH level among COPD patients of coal mine site; while in case of control subjects the observed levels of circulating heavy metals (Cd, Pb, Hg, and Cr) may not have such effects.

3.3. Effect of cadmium on intracellular ROS production, GST activity, GSH level and the protein expression of NOX4, Nrf2, GCLC, and GST in THP-1 monocytes

Using THP1 monocyte cell culture model, we have further investigated the direct effect of Cd on intracellular ROS production, GST activity, GSH level and the protein expression of the associated signaling molecules, namely NOX4, Nrf2, GCLC, and GST (Fig. 7). Results demonstrate that treatment of monocytes with $CdCl_2$ (as a source of Cd) at doses of 0.004, 0.008, 0.016, and 0.032 μg/mL significantly ($p < .05$) increased ROS production and the protein expression of NOX4, while decreased the expression of GST protein compared to the untreated group. Treatment of monocytes with Cd also decreased the protein expression of Nrf2 and GCLC and reduced the GST activity and GSH level compared to control.

3.4. Effect of lead on ROS production, GST activity, GSH level and expression of NOX4, Nrf2, GCLC, and GST in THP-1 monocytes

Fig. 8 represents the direct effect of Pb onthe protein expression of NOX4, Nrf2, GCLC, and GST protein, as well as ROS production, GST activity, and GSH level in THP-1 monocytes. Results demonstrate that the levels of ROS production and the expression of NOX4 protein were significantly ($p < .05$) higher, and Nrf2 was lower in the $PbCl_2$ (as a source of Pb) treated group (0.003–0.048 μg/mL) compared to control group. Likewise, the exposure of Pb also caused a significant ($p < .05$) dose-dependent decrease in the protein expression of GCLC and GST and intracellular GST activity and GSH level compared to the untreated group.

3.5. Effect of mercury on ROS production, GST activity, GSH level and expression of NOX4, Nrf2, GCLC, and GST in THP-1 monocytes

Dose-dependent effects of Hg on the protein expression of NOX4, Nrf2, GCLC, and GST, intracellular ROS production, GST activity and GSH level in monocytes are shown in Fig. 9. Results show that treatment of $HgCl_2$ (as a source of Hg) at doses of 0.016, 0.032, and 0.064 μg/mL significantly ($p < .05$) increased the NOX4 protein expression and ROS production while decreased the expression of GST protein. However, the expression of Nrf2 and GCLC proteins and intracellular GST activity and GSH level were decreased significantly ($p < .05$) upon treatment with Hg in a dose-dependent manner.

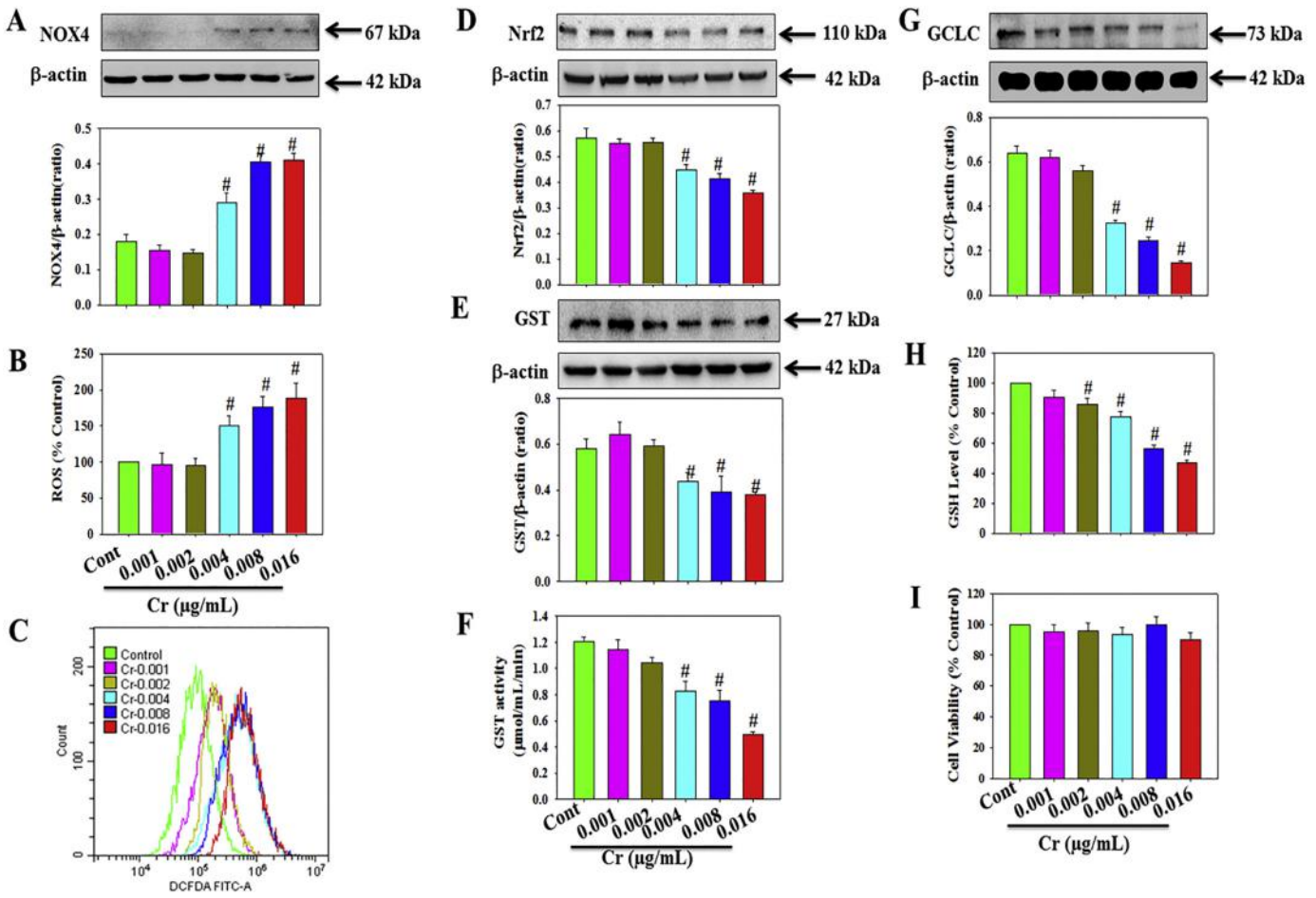

Fig. 10. Effect of Cr on NOX4 protein expression (A), intracellular ROS level using fluorescence spectroscopy (B) and Flowcytometry (C), Nrf2 protein expression (D), GST protein expression (E), GST activity (F), GCLC protein expression (G), GSH level (H), and Cell viability (I) in THP-1 monocyte cells. Cells were pre-treated with increasing doses (0.001–0.016 μg/mL) of $CrCl_3$ (as a source of chromium) for 24 h at 37 °C. Values are mean ± standard error. "#" denotes the significance difference from control group ($p\# < 0.05$).

3.6. Effect of chromium on ROS production, GST activity, GSH level and expression of NOX4, Nrf2, GCLC, and GST in THP-1 monocytes

Fig. 10 demonstrate the dose dependent effect of Cr-treatment on the protein expression of NOX4, Nrf2, GCLC, and GST, and the levels of ROS production, GST activity and GSH level in monocytes. It was observed that treatment of $CrCl_3$ (as a source of Cr) at doses of 0.004, 0.008 and 0.016 μg/mL significantly ($p < .05$) increased the NOX4 protein expression and intracellular ROS production, while decreased the expression of Nrf2, GST and GCLC protein as well as GST activity and GSH level compared to the untreated control.

Different doses of Cd, Pb, Hg, and Crdid not cause any change in cell viability compared to control. All these cell culture studies suggest a critical role of heavy metals in the generation oxidative stress and cellular antioxidant imbalance via down-regulating the GST activity and GSH level by impairing the NOX4/Nrf2/GCLC/GST signaling pathway.

4. Discussion

In recent years, there has been an increasing global health-care concern associated with occupational exposure to heavy metals (Jang, 2012; Moitra et al., 2013; Tchounwou et al., 2012). Moreover, COPD is one of the major disease burden globally associated with occupational exposure (May and Li, 2015). However, the information on the mechanism of circulating heavy metal mediated development of COPD with respect to the occupational exposure remains unclear.

The present study for the first time showed that the blood levels of heavy metals (Cd, Pb, Hg, and Cr) were significantly higher among COPD patients of coal mine site compared to control subjects. In addition, a significant negative correlation was also observed between blood levels of these heavy metals and lung function of COPD patients. Earlier, Korean National Health and Nutrition Examination Survey IV–V reported an inverse relationship of serum lead and cadmium levels with lung function (FEV_1/FVC) in a non-institutionalized civilian Korean population (Leem et al., 2015). Similarly, Rokadia et al. have found a positive relationship of blood cadmium and lead concentrations with the risk of developing obstructive lung diseases in US population (Rokadia and Agarwal, 2013). Furthermore, higher blood level of lead was observed among workers of Velenje coal mine in Slovenia, based on exposure time compared to the control (Zimet et al., 2017). In agreement with these earlier studies, our present investigation also demonstrated the role of heavy metal exposure as a critical risk factor in pathogenesis of COPD.

Impairment of oxidant-antioxidant system leads to the development of oxidative stress, which plays an important role in COPD pathophysiology (Mak, 2008). Pinho et al. observed an increase in systemic and pulmonary oxidative stress markers among COPD patients (Pinho et al., 2007). GST and GSH are two primary antioxidant molecules which play an important role in the reduction of cellular oxidative stress (Li, 2011). Evelo et al. reported the lower GST activity and GSH level with reference to the early stages of pneumoconiosis among the workers of

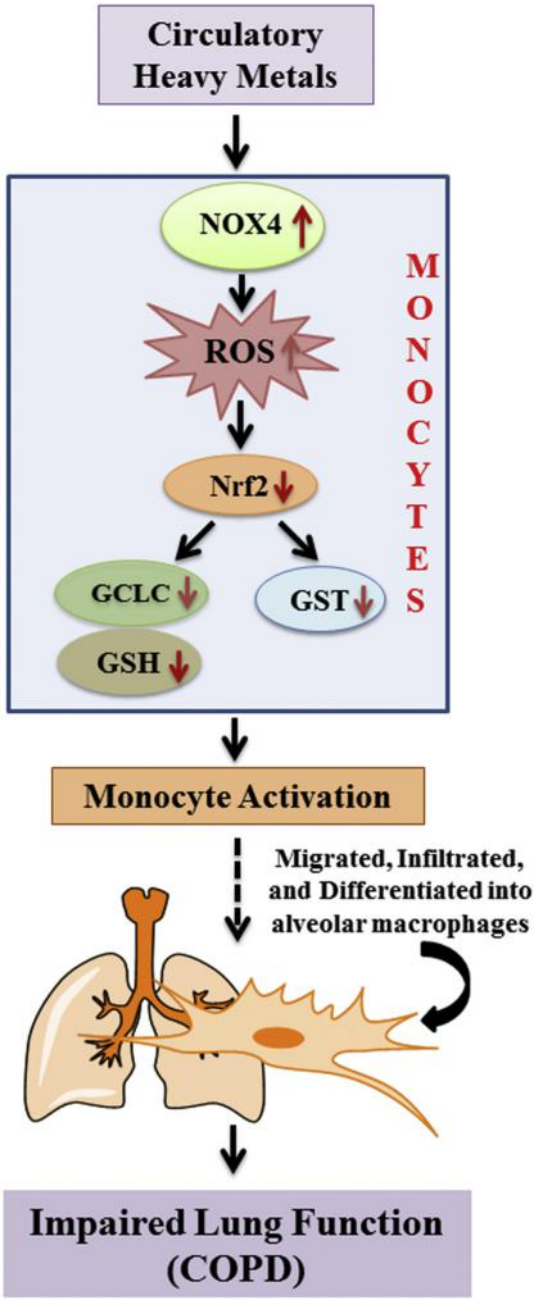

Fig. 11. Schematic representation of the proposed mechanism underlying the role of heavy metals-mediated lung function impairment leading to COPD.

Belgian coal mine (Evelo et al., 1993). Similarly, Junior et al. reported lower GSH level among coal dust exposed groups compared to control (Junior et al., 2009). Synthesis of cellular GSH is controlled by glutamate cysteine ligase (GCL) enzyme, comprising of catalytic subunit (GCLC) and modifier subunit (GCLM) (Chen et al., 2005). Cheng et al. further reported the down-regulation of GCLC mRNA expression and lower plasma GSH level among smoker COPD group (Cheng et al., 2016). The present study demonstrated the lower GST activity and GSH level in the plasma samples collected from COPD patients of coalmine site compared to the control. Interestingly, a significant negative correlation was observed between the blood levels of heavy metals (Cd, Pb, Cr, and Hg) and plasma GST activity as well as GSH level among COPD patients.

Among various cell types, circulatory monocytes play an important role in COPD (Yang et al., 2018). Under pathological condition, blood monocytes from the circulatory pool migrate into lungs and then infiltrated into alveolar space to form alveolar macrophages, which drive the inflammatory processes via releasing various chemotactic factors and recruiting neutrophils (Barnes, 2004). It was observed that in bronchoalveolar-lavage of patients with COPD, the macrophages numbers were 5 to 10 times higher than those seen in control (Barnes, 2000). Alveolar-macrophage derived metalloproteinases caused the inflammation via releasing TNF-α followed by neutrophil influx, endothelial activation, and tissue breakdown (Aldonyte et al., 2003). Using THP-1 monocyte cells, this study further reported that treatment with individual heavy metals, such as cadmium, lead, chromium, and mercury, dose-dependently decreased the intracellular GST activity, GSH level, and increased ROS production. In addition, treatment with all these heavy metals also down regulated the protein expression of GST and GCLC. Combining these data, the present study suggests a direct role of heavy metals in development of oxidative stress and impairment of GST and GSH antioxidant system in monocytes, which may be linked to the development of COPD.

NOX4 and Nrf2 are two important enzyme systems proactively associated with the regulation of oxidant-antioxidant responses (Kuroda et al., 2010; Nguyen et al., 2009). Findings of earlier studies suggested that NOX mediated signaling is critical in activation of Nrf2 (Brewer et al., 2011; Papaiahgari et al., 2004). Recent experimental evidence showed that NOX4 expression is negatively regulated by activation of Nrf2in endothelial cells (Goettsch et al., 2011). However, another study using both mouse and human lung endothelial cells has demonstrated the role of Nrf2 in up-regulation of NOX4 expression in response to hyperoxia (Pendyala et al., 2011). The present study demonstrated that treatment with heavy metals (Cd, Pb, Cr and Hg) dose-dependently up-regulated the NOX4 protein expression and down-regulated Nrf2 protein expression in THP-1 monocyte cells, which may be linked to the heavy metal induced decrease in GST activity and GSH levels, a critical risk factor in the COPD pathophysiology.

In conclusion, the present study demonstrates that the circulatory heavy metals (Cd, Pb, Hg, and Cr) were significantly higher among COPD patients living near the coal mine site of Jaintia hill, Meghalaya and also significantly and inversely associated with lung function, plasma GST activity, and GSH level. Using monocyte cell culture model, this study further showed that treatment with individual heavy metals (Cd, Pb, Hg, and Cr) dose-dependently increased the NOX4 protein expression and ROS production and decreased the Nrf2, GST, and GCLC protein expression, GST activity, and GSH level. Combining all, the present study suggested that the circulatory heavy metals in COPD patients of coal mine site may play a crucial role in lung function decline, down-regulating plasma GST activity and GSH level via impairing the NOX4/Nrf2/GCLC/GST signaling pathway in monocytes, which may cause monocyte activation, migration, differentiation into alveolar macrophages, and initiate the COPD pathophysiology (Fig. 11). These findings of the link between circulatory heavy metals, monocytes, oxidative stress, NOX4/Nrf2/GCLC/GST signaling pathway, impair GST activity and GSH level, and decline lung function in COPD patients may be helpful for the development of a novel adjuvant therapy to achieve better control of COPD and improve the lives of the patient population.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References

Agrawal, S., Flora, G., Bhatnagar, P., Flora, S.J., 2014. Comparative oxidative stress, metallothionein induction and organ toxicity following chronic exposure to arsenic, lead and mercury in rats. Cell Mol Biol (Noisy-le-grand) 60, 13–21.

Aldonyte, R., Jansson, L., Piitulainen, E., Janciauskiene, S., 2003. Circulating monocytes from healthy individuals and COPD patients. Respir. Res. 4, 1–8.

Almeida Lopes, A.C.B., Urbano, M.R., Souza-Nogueira, A., Oliveira-Paula, G.H., Michelin, A.P., Carvalho, M.F.H., Camargo, A.E.I., Peixe, T.S., Cabrera, M.A.S., Paoliello, M.M.B., 2017. Association of lead, cadmium and mercury with paraoxonase 1 activity and malondialdehyde in a general population in Southern Brazil. Environ. Res. 156, 674–682.

Barnes, P.J., 2000. Chronic obstructive pulmonary disease. N. Engl. J. Med. 343, 269–280.

Barnes, P.J., 2004. Mediators of chronic obstructive pulmonary diseases. Pharmacol. Rev. 56, 515–548.

Boschetto, P., Quintavalle, S., Miotto, D., Lo Cascio, N., Zeni, E., Mapp, C.E., 2006. Chronic obstructive pulmonary disease (COPD) and occupational exposures. J. Occup. Med. Toxicol 1, 11.

Brewer, A.C., Murray, T.V., Arno, M., Zhang, M., Anilkumar, N.P., Mann, G.E., Shah, A.M., 2011. Nox4 regulates Nrf2 and glutathione redox in cardiomyocytes in vivo. Free Radic. Biol. Med. 51, 205–215.

Cao, T., Xu, N., Wang, Z., Liu, H., 2017. Effects of glutathione S-transferase gene polymorphisms and antioxidant capacity per unit albumin on the pathogenesis of chronic obstructive pulmonary disease. Oxidative Med. Cell. Longev. 2017, 8.

Chen, Y., Shertzer, H.G., Schneider, S.N., Nebert, D.W., Dalton, T.P., 2005. Glutamate cysteine ligase catalysis: dependence on ATP and modifier subunit for regulation of tissue glutathione levels. J. Biol. Chem. 280, 33766–33774.

Cheng, L., Liu, J., Li, B., Liu, S., Li, X., Tu, H., 2016. Cigarette smoke-induced hypermethylation of the GCLC gene is associated with COPD. Chest 149, 474–482.

Clinton, O.E., 1977. Determination of selenium in blood and plant material by hydride generation and atomic-absorption spectroscopy. Analyst 102, 187–192.

Dey, T., Gogoi, K., Unni, B.G., Kalita, M., Bharadwaz, M., Bhattacharjee, M., Boruah, P.K., Bora, T., Ozah, D., 2014. Role of glutathione S transferase polymorphism in COPD with special reference to peoples living in the vicinity of the open cast coal mine of Assam. PLoS One 9, e96739.

Dey, T., Dutta, P., Manna, P., Kalita, J., Boruah, H.P.D., Buragohain, A.K., Unni, B., Ozah, D., Kumar Goswami, M., Kotokey, R.K., 2016. Cigarette smoke compounds induce cellular redox imbalance, activate NF-[small kappa]B, and increase TNF-[small alpha]/CRP secretion: a possible pathway in the pathogenesis of COPD. Toxicol. Res 5, 895–904.

Elmasry, S.A., Al-Azzawi, M.A., Ghoneim, A.H., Nasr, M.Y., AboZaid, M.M.N., 2015. Role of oxidant–antioxidant imbalance in the pathogenesis of chronic obstructive pulmonary disease. Egypt. J. Chest Dis. Tuberc 64, 813–820.

Evelo, C.T., Bos, R.P., Borm, P.J., 1993. Decreased glutathione content and glutathione S-transferase activity in red blood cells of coal miners with early stages of pneumoconiosis. Br. J. Ind. Med. 50, 633–636.

Goettsch, C., Goettsch, W., Brux, M., Haschke, C., Brunssen, C., Muller, G., Bornstein, S.R., Duerrschmidt, N., Wagner, A.H., Morawietz, H., 2011. Arterial flow reduces oxidative stress via an antioxidant response element and Oct-1 binding site within the NADPH oxidase 4 promoter in endothelial cells. Basic Res. Cardiol. 106, 551–561.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.

Heo, J., Park, H.S., Hong, Y., Park, J., Hong, S.-H., Bang, C.Y., Lim, M.-N., Kim, W.J., 2017. Serum heavy metals and lung function in a chronic obstructive pulmonary disease cohort. Toxicol. Environ. Heal. Sci. 9, 30–35.

Ishii, T., Matsuse, T., Teramoto, S., Matsui, H., Miyao, M., Hosoi, T., Takahashi, H., Fukuchi, Y., Ouchi, Y., 1999. Glutathione S-transferase P1 (GSTP1) polymorphism in patients with chronic obstructive pulmonary disease. Thorax 54, 693–696.

Ishii, Y., Itoh, K., Morishima, Y., Kimura, T., Kiwamoto, T., Iizuka, T., Hegab, A.E., Hosoya, T., Nomura, A., Sakamoto, T., Yamamoto, M., Sekizawa, K., 2005. Transcription factor Nrf2 plays a pivotal role in protection against elastase-induced pulmonary inflammation and emphysema. J. Immunol. 175, 6968–6975.

Jang, A.S., 2012. Particulate air pollutants and respiratory diseases. In: Haryanto, B. (Ed.), Air Pollution a Comprehensive Perspective, (In Tech Open).

Jiang, Z., Li, D., Zhu, L., 2017. The distinct roles of circulating monocytes and alveolar macrophages in mouse model with acute lung injury. J. Pulm. Respir. Med 7.

Junior, S.A., Possamai, P.F., Budni, P., Backes, P., Parisotto, E.B., Rizelio, V.M., Torres, M.A., Colepicolo, P., Wilhelm, F.D., 2009. Occupational airborne contamination in south Brazil: 1. Oxidative stress detected in the blood of coal miners. Ecotoxicology 18, 1150–1157.

Kraim-Leleu, M., Lesage, F.X., Drame, M., Lebargy, F., Deschamps, F., 2016. Occupational risk factors for COPD: a case-control study. PLoS One 11, e0158719.

Kuroda, J., Ago, T., Matsushima, S., Zhai, P., Schneider, M.D., Sadoshima, J., 2010. NADPH oxidase 4 (Nox4) is a major source of oxidative stress in the failing heart. Proc. Natl. Acad. Sci. 107, 15565.

Lakhdar, R., Denden, S., Knani, J., Leban, N., Daimi, H., Hassine, M., Lefranc, G., Chibani, J.B., Khelil, A.H., 2011. Combined analysis of EPHX1, GSTP1, GSTM1 and GSTT1 gene polymorphisms in relation to chronic obstructive pulmonary disease risk and lung function impairment. Dis. Markers 30, 253–263.

Leem, A.Y., Kim, S.K., Chang, J., Kang, Y.A., Kim, Y.S., Park, M.S., Kim, S.Y., Kim, E.Y., Chung, K.S., Jung, J.Y., 2015. Relationship between blood levels of heavy metals and lung function based on the Korean National Health and Nutrition Examination Survey IV-V. Int. J. Chron. Obstruct. Pulmon. Dis 10, 1559–1570.

Li, X., 2011. Glutathione and Glutathione-S-Transferase in Detoxification Mechanisms, General, Applied and Systems Toxicology.

Little, B.B., Ignasiak, Z., Slawinska, T., Posluszny, P., Malina, R.M., Wiegman, D.L., 2017. Blood lead levels, pulmonary function and agility in Polish schoolchildren. Ann. Hum. Biol. 44, 723–728.

Liu, Xianyan, Hao, Binwei, Ma, Ailing, He, Jinxi, Liu, Xiaoming, Chen, Juan, 2016. The expression of NOX4 in smooth muscles of small airway correlates with the disease severity of COPD. Biomed. Res. Int. 1–17.

Loboda, A., Damulewicz, M., Pyza, E., Jozkowicz, A., Dulak, J., 2016. Role of Nrf2/HO-1 system in development, oxidative stress response and diseases: an evolutionarily conserved mechanism. Cell. Mol. Life Sci. 73, 3221–3247.

Mak, J.C., 2008. Pathogenesis of COPD. Part II. Oxidative-antioxidative imbalance. Int. J. Tuberc. Lung. Dis 12, 368–374.

Manna, P., Jain, S.K., 2014. Effect of PIP3 on adhesion molecules and adhesion of THP-1 monocytes to HUVEC treated with high glucose. Cell. Physiol. Biochem. 33, 1197–1204.

May, S.M., Li, J.T.C., 2015. Burden of chronic obstructive pulmonary disease: healthcare costs and beyond. Allergy Asthma Proc 36, 4–10.

Mohammed, A., Gutta, V., Ansari, M.S., Saladi Venkata, R., Jamil, K., 2017. Altered antioxidant enzyme activity with severity and comorbidities of chronic obstructive pulmonary disease (COPD) in South Indian population. COPD Res. Pract 3, 4.

Moitra, S., Blanc, P.D., Sahu, S., 2013. Adverse respiratory effects associated with cadmium exposure in small-scale jewellery workshops in India. Thorax 68, 565–570.

Nebert, D.W., Vasiliou, V., 2004. Analysis of the glutathione S-transferase (GST) gene family. Hum. Genomics 1, 460–464.

Nguyen, T., Nioi, P., Pickett, C.B., 2009. The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J. Biol. Chem. 284, 13291–13295.

Oh, C.M., Oh, I.H., Lee, J.K., Park, Y.H., Choe, B.K., Yoon, T.Y., Choi, J.M., 2014. Blood cadmium levels are associated with a decline in lung function in males. Environ. Res. 132, 119–125.

Papaiahgari, S., Kleeberger, S.R., Cho, H.Y., Kalvakolanu, D.V., Reddy, S.P., 2004. NADPH oxidase and ERK signaling regulates hyperoxia-induced Nrf2-ARE transcriptional response in pulmonary epithelial cells. J. Biol. Chem. 279, 42302–42312.

Patra, R.C., Rautray, A.K., Swarup, D., 2011. Oxidative stress in lead and cadmium toxicity and its amelioration. Vet. Med. Int 2011, 457327.

Pendyala, S., Moitra, J., Kalari, S., Kleeberger, S.R., Zhao, Y., Reddy, S.P., Garcia, J.G., Natarajan, V., 2011. Nrf2 regulates hyperoxia-induced Nox4 expression in human lung endothelium: identification of functional antioxidant response elements on the Nox4 promoter. Free Radic. Biol. Med. 50, 1749–1759.

Pinho, R.A., Chiesa, D., Mezzomo, K.M., Andrades, M.E., Bonatto, F., Gelain, D., Dal Pizzol, F., Knorst, M.M., Moreira, J.C.F., 2007. Oxidative stress in chronic obstructive pulmonary disease patients submitted to a rehabilitation program. Respir. Med. 101, 1830–1835.

Rehman, K., Fatima, F., Waheed, I., Akash, M.S.H., 2018. Prevalence of exposure of heavy metals and their impact on health consequences. J. Cell. Biochem 119, 157–184.

Rodriguez, F., de la Roza, C., Jardi, R., Schaper, M., Vidal, R., Miravitlles, M., 2005. Glutathione S-transferase P1 and lung function in patients with alpha1-antitrypsin deficiency and COPD. Chest 127, 1537–1543.

Rokadia, H.K., Agarwal, S., 2013. Serum heavy metals and obstructive lung disease: results from the National Health and Nutrition Examination Survey. Chest 143, 388–397.

Shukla, R.K., Kant, S., Bhattacharya, S., Mittal, B., 2011. Association of genetic polymorphism of GSTT1, GSTM1 and GSTM3 in COPD patients in a north Indian population. COPD: J. Chron. Obstruct. Pulmon. Dis. 8, 167–172.

Tchounwou, P.B., Yedjou, C.G., Patlolla, A.K., Sutton, D.J., 2012. Heavy metal toxicity and the environment. EXS 101, 133–164.

Troiano, J.A., Potje, S.R., Graton, M.E., Cavalari, P., Pereira, A.A., Vale, G.T., Nakamune, A.C., Sumida, D.H., Tirapelli, C.R., Antoniali, C., 2016. Decreased reactive oxygen species production and NOX1, NOX2, NOX4 expressions contribute to hyporeactivity to phenylephrine in aortas of pregnant SHR. Life Sci. 144, 178–184.

Yang, J., Qiao, M., Li, Y., Hu, G., Song, C., Xue, L., Bai, H., Yang, J., Yang, X., 2018. Expansion of a population of large monocytes (atypical monocytes) in peripheral blood of patients with acute exacerbations of chronic obstructive pulmonary diseases. Mediat. Inflamm. 2018, 1–13.

Zimet, Z., Bilban, M., Fabjan, T., Kumer, K., Poljšak, B., Osredkar, J., 2017. Lead Exposure and Oxidative Stress in Coal Miners.