3.1. Selection and description of study area

The following study areas were selected for soil, water and food crops collection: i) Agricultural area: (AA): Comprising agricultural parts of Jhunsi, which is the eastern part of Allahabad District, close to Rivers Ganga and Yamuna, ii) Industrial area (IA): Comprising industrial areas of Naini, which is the southern part of Allahabad District, also close to the Rivers Ganga and Yamuna iii) Coal mining area (CMA): Comprising the Singrauli coal mines, which lie partly in the Singrauli district of Madhya Pradesh and partly in Sonebhadra district of Uttar Pradesh. The areas of study are mapped in figure 3.1. Two areas representing agricultural and industrial parts of Allahabad district (25°28’ N latitude, 81°54’ E longitude; 98 m above mean sea level) were selected because Allahabad is one of the least industrialized and least polluted cities in east Uttar Pradesh (UP) (Miyazaki, 2006). Naini is the chief industrial area of Allahabad, which has or used to have several polluting industries such as glass, structural, steel, energy, and chemical works (Miyazaki, 2006). Singrauli Coal Mines (24° 00' to 24° 15' N latitudes and 82° 30' to 82°45' E longitudes), spread over a total area of 2201 km², of which at present only a 300 km² area is exploited for coal (Javed and Khan, 2012), were selected because they have been declared as the country’s ninth most critically polluted area by the Ministry of Environment (CSE, 2012) and are the coal mines closest to Allahabad.
3.2. Selection and collection of soil, water, and food crop samples

Sampling was conducted in the months of January and February, 2011, at all the selected locations. Soils were collected from the upper 15 cm of the soil profile. Three random samples on each plot were taken from all selected locations namely AA, IA, and CMA. At the same time, vegetable samples were collected on the plot where a soil sample was taken. Reference soil sample from the three locations were also collected which had no agricultural activity at collection time. Samples of soil and food crops were stored in polyethylene bags in the field and transferred to the laboratory. Food crops included edible parts of spinach (Spinacia oleracea), potato (Solanum tuberosum), rice (Oryza sativa), wheat (Triticum aestivum), and maize (Zea mays). Three sources of water samples, surface water (SW), ground water (GW), and tap water (TW) were collected. Surface water is the water more likely to contain surface contaminants and is used for irrigation purposes; groundwater was obtained comprised deep water below the water table, obtained from tube wells, which was being used to irrigate the fields, and tap water, which had undergone some treatment and was obtained from municipal taps. 100 mL samples of each source from the three locations were collected in triplicate in pre-acid washed polypropylene bottles and
1 ml of concentrated HNO₃ was added to the water sample to prevent microbial activity. All samples were brought to the laboratory and kept in a refrigerator before digestion.

3.3. Estimation of physico-chemical properties of

3.3.1. Soil
The pH, electrical conductivity, moisture content, water holding capacity (WHC), bulk density, organic matter, were analyzed in S₁, S₂, S₃ and S₄ collected from AA, IA and CMA. The pH and electrical conductivity were measured in a soil suspension (1:10 w/v dilution) by digital pH meter (labotronics-LT-1) and conductivity meter (Systronics-304), respectively. Bulk density was calculated following Blake and Hartge, (1986). Moisture content and water holding capacity was computed following Saxena, (1987). Organic matter was examined by Potassium dichromate titration method (Saxena, 1987).

3.3.2. Water
The pH, electrical conductivity, total hardness, total dissolved solids (TDS), turbidity, temperature, dissolved oxygen (DO), were analyzed following the standard methods described in APHA, (2005).

3.4. Estimation of moisture content of soil and different parts of food crops
The moisture content (also referred to as water content) is the ratio of the mass of water in a sample to the mass of solids in the sample, expressed as a percentage. Moisture content was analyzed gravimetrically following Saxena, (1987). Samples (100 g) were taken in triplicate, and dried to a constant weight in an oven at 70 °C for 24 h, cooled in a desiccator and the difference in weight recorded for two consecutive days. Moisture content is the per cent sample weight lost in drying. The moisture contents of all soil and crops studied were computed by drying them in an oven till constant weight. Edible parts of each vegetable and cereal crop were weighed after washing with de-ionized water and blotting dry with tissue paper.

\[
\text{Moisture Content (\%)} = \frac{\text{Wet weight} - \text{dry weight}}{\text{Wet weight}} \times 100
\]
Figure 3.2. Oven dried cereals for moisture content determination.

Figure 3.3. Oven dried spinach roots and leaves for moisture content determination.

Figure 3.4. Oven dried potato roots, leaves and tuber for moisture content determination.
3.5. Digestion of soil and food crop samples

Plant samples (1 g) were digested with 15 ml of tri-acid mixture (HNO$_3$, H$_2$SO$_4$, and HClO$_4$ in 5:1:1 ratio) at 80°C until a transparent solution was obtained (Allen et al., 1986). Soil samples were air dried, crushed, and passed through a 2-mm mesh size sieve and dried in an oven to calculate moisture content. Digestion of soil was performed in the same way as for plant samples. After cooling, the digested soil and edible plant sample was filtered using Whatman No. 42 filter paper and the filtrate was finally made up to 50 ml with distilled water and stored in a Tarson sample container (50 ml).

3.6. Digestion of water samples

Water samples (50 ml) were digested with 10 ml of concentrated HNO$_3$ at 80°C until the solution became transparent (APHA, 2005) to remove organic matter. The solution was filtered through Whatman No. 42 filter paper and the total volume was made up to 50 ml with distilled water.

3.7. Estimation of heavy metals by AAS (Atomic Absorption Spectrophotometer)

Concentrations of Pb, Cd, Cr, Zn, Fe, Cu, and Co in the filtrate of digested soil, edible plant samples, and water samples were estimated by using an atomic absorption spectrophotometer (AAS) (Perkin Elmer AAS-3100). The instrument was fitted with specific lamps for particular metal. Standard stock solutions of 1000 ppm for all the metals were obtained from Sisco Research Laboratories Pvt. Ltd., India. These solutions were diluted to appropriate concentrations to calibrate the instrument.

3.8. Data Analysis

3.8.1. Calculation of Transfer Factor (TF)

The ability of a metal species in its different forms to migrate from the soil through the plant parts and make itself available for consumption was represented by the Transfer Factor (TF), which was calculated to understand the heavy metal accumulation in edible portion of cereals and vegetables following Cui et al., (2004), as follows:

\[
\text{Transfer Factor} = \frac{\text{Concentration of metal in edible portion of test food (dry weight basis)}}{\text{Concentration of metal in soil (dry weight basis)}}
\]
3.8.2. Calculation of oral intake or daily intake of metals from vegetables cereals and water (DIM)

The daily oral intake of metals, transferred from soil through vegetables, cereals, or water was calculated as described by (Khan et al., 2008b) by the following formula:

\[
\text{Daily Intake of Metal (DIM)} = \frac{(C_{metal} \times C_{factor} \times D_f)}{B_{average bodyweight}}
\]

Where \(C_{metal}\) represents the heavy metal concentrations in the edible part of a plant (wet weight in mg/kg), \(C_{factor}\) represents the conversion factor, \(D_f\) represents daily intake of a particular foodstuff and \(B_{average bodyweight}\) represents the average bodyweight in kg.

In the present study, foodstuffs analyzed were spinach, potato, rice, and wheat, representing leafy vegetables, tubers, and cereals, respectively. Concentrations of metals were measured in ash. However, to compute the Daily Intake of Metal (DIM), this was converted to wet weight concentrations. The moisture contents of all crops studied were computed by drying them in an oven till constant weight, as described previously. The moisture contents of all crops studied are summarized in table 4.3.1 in results section and used to derive Conversion Factors for each crop, where Conversion factor = (100 - percent moisture content)/percent moisture content.

For computation of \(D_f\), intakes were based on the Recommended Dietary Allowances (RDA) given by the Indian Council of Medical Research (ICMR, 2010). For food groups of cereals, leafy vegetables, and tubers, ICMR has recommended a daily intake of 450 g, 100 g, and 200 g respectively for a balanced diet to meet the nutritional requirements for a moderately active reference man weighing 60 kg. Drinking water also contributes to metal intake. Hence, DIM was also computed for water, assuming an average intake of 2 litres of tap water (ICMR, 2010). For \(B_{average bodyweight}\), the actual mean bodyweights of 66.6, 64.3, and 63 kg, respectively for AA, IA and CMA areas were used for computation.

3.8.3. Calculation of Health Risk Index (HRI) due to metal contamination of vegetable, cereals and water

The Health Risk Index (HRI) was computed as described by USEPA, (2002). The HRI through consumption of cereals, vegetables and water (tap) was assessed as the
ratio of DIM (mg per kg bodyweight/day) and the reference oral dose (RfD) for each metal.

\[ \text{HRI} = \frac{\text{DIM}}{\text{RfD}} \]

Reference oral doses (RfDs) for Pb, Cr, Co, Cu, Zn, Fe, and Cd were respectively 0.004, 1.5, 0.02, 0.04, 0.3, 0.3, and 0.001 mg/ kg/ day (USEPA, 2006). An index >1 is categorized not safe for human health (USEPA, 2002). Iron, Zn, Cu, Co, and Cr are dietary essential metals for which recommended intakes (ICMR, 2010) have been recognized, but that may have detrimental effects when present in doses exceeding RfD. Hence their HRI was also computed.

The next step was to assess the concentrations of essential and contaminating metals in blood of coal mining exposed respondents and comparison with Control respondents not similarly exposed. For the purpose, various study design were explored.

3.9. Selection of respondents for biochemical studies

Respondents were selected from coal mining area (CMA) as CME (coal mining exposed) group as they had frequent and long term exposure to mining activities and they were compared with residents from AA and IA who served as control because they had no exposure to mining activities. As the migration of population is significant, samples were collected from residents only if their period of residence in the community was more than five years. The two groups were matched for age and body mass index which was computed as

\[ \text{BMI} = \frac{\text{body weight (Kg)}}{\text{height (m^2)}} \]

3.9.1. Inclusion and exclusion criteria

Inclusion criteria were defined to include respondents of working age group. The controls were respondents having no exposure to coal mining while coal mining exposed had long term exposure to mining activities. Those who met these criteria were further screened for exclusion criteria, which were defined to exclude those suffering from obesity, hypertension, dyslipidemia, hyperglycemia, systemic disorders of cardiovascular, central nervous and respiratory systems and addiction to alcohol and smoking.
These conditions were self reported or had been diagnosed by physicians on the basis of blood tests and other routine diagnostic procedures. For this cross sectional study, about 200 male each from control and coal mining area were screened for inclusion, exclusion criteria. 60 respondents were enrolled as Controls and 63 as coal mining exposed group (CME). The objective of the study was explained to them and informed consent was obtained. Informed consent was taken and questionnaire (attached as Annexure 1) was developed for the purpose was filled, as far as possible. Their weight and height were measured using standard techniques, ensuring reliability as far as possible. Blood of all participants was collected by trained technicians for biochemical analysis.

The protocol of the study was approved by the Institutional Ethics Committee (IEC) of Population Resource & Research Centre (PRRC), Allahabad, (attached as Annexure 2) and informed consent to participate in the study was obtained from all participants. Venous blood sample was drawn and collected and brought to laboratory for further investigations.

3.9.2. Study Design
Several study designs are available for studies on population, such as epidemiological, cohort, case-control, occupational epidemiological and cross-sectional studies. To study the effect of pollution in coal-mining areas it was required to study a group exposed to pollution of mines and a group having no exposure to pollution. Hence it was decided to adopt the case-control study design since a case control study compares patients who have a disease or outcome of interest (cases) with patients who do not have the disease or outcome (controls), and looks back retrospectively to compare how frequently the exposure to a risk factor is present in each group to determine the relationship between the risk factor and the disease.

In a case-control study, it is imperative that the investigator has explicitly defined inclusion and exclusion criteria prior to the selection of cases. Statistical adjustment may be rendered more efficient by matching cases and controls for exposure to confounders, either on an individual basis (for example by pairing each case with a control of the same age and sex) or in groups (for example, choosing a control group
with an overall age and sex distribution similar to that of the cases). Based on these considerations, respondents were selected.

3.10. Processing of blood sample for biochemical estimations

6 ml of venous blood was drawn of which 1 ml was collected in EDTA vial for Hb and Hct estimation and 4 ml into Acid-Citrate-Dextrose (ACD) and kept on ice for not more than 1 hour before processing. 1 ml was collected in boiling tube for AAS metal analysis which is discussed below. The samples were centrifuged at 3000 rpm for 5 min, plasma was collected and red blood cells (RBCs) were washed three times with normal saline. RBC was used to prepare 1:20 hemolysate. Packed RBCs obtained were suspended in approximately 1 volume of 0.154 M NaCl. To 0.2 ml of this suspension, 1.8 ml of β-merceptoethanol-EDTA stabilizing solution (0.05 ml of β-merceptoethanol and 10 ml of neutralized 10% EDTA in 1l (distilled water) was added. Plasma and 1:20 hemolysate were transferred into separate eppendorf tubes and stored at -80°C, until analysis. Processing of blood sample is given in figure 3.5. Specific investigations were done in various parts of blood:

- **Whole blood**- Hemoglobin, Hct.
- **Plasma**- Ferric Reducing Ability of Plasma (FRAP), Glutathione peroxidase (GPx), Ferritin, Plasma iron, Total Iron Binding Capacity (TIBC), Uric Acid (UA), Protein.
- **Hemolysate**- Malonaldialdehyde (MDA), Superoxide Dismutase (SOD).

3.11. Processing of blood sample for analysis of heavy metals

Iron, Zn, Cu, Co, Cd, Cr and Ca were analyzed in blood by flame atomic absorption spectrophotometer (AAS) (Perkin Elmer AAS-3100) using respective hollow cathode lamps (248.3 nm). To 1 ml of blood in a boiling tube 2 ml of 1:1 mixture of concentrated HNO₃ and HCLO₄ was added. The samples were digested at 60°C for 1 hour and then at 120-150°C for 1 hour in water bath. Digestion duration might be increased until a clear solution was obtained. Volume was raised to 10 ml with double distilled water and fed to AAS for elemental analysis (Ahamed et al., 2007). Blood lead was determined using a graphite furnace atomic absorption spectrometer (283.3 nm). 50 μl of blood was diluted with diluents in1:10. The diluents [Triton X-100, 0.1% (w/v); NH₄H₂PO₄ 0.2% (w/v); NH₃ (0.14 mol/l)] was prepared in deionized water (Ahamed et al., 2007).
3.12. Biochemical estimations

3.12.1. Assessment of iron status:

3.12.1.1. Hemoglobin (Hb)

Hemoglobin was estimated as cyanmethemoglobin in blood using Drabkin’s reagent by following recommendations from International Committee for Standardization in Haematology, ICSH (1967). For this, 0.01 ml of blood of test sample was added to 5 ml of Drabkin reagent, a solution containing ferricyanide and cyanide. Simultaneously, 0.01 ml of Hb standard (15.06 g/dl) was also added to 5 ml of Drabkin solution. Absorbance of test sample and Hb standard were taken against Drabkin solution at 540 nm and Hb (g/dl) was calculated as below:

\[
\text{Hb (g/dl) = (Absorbance of test/Absorbance of standard) \times 15.06}
\]

Figure 3.5. Flow chart of processing of blood sample.
3.12.1.2. Packed Cell Volume (Hct)

Wintrobe tubes were used to estimate the Hct, 0.2 ml blood was added to the tube and allowed to stand for 2-4 hours, after the incubation period blood cells settled at the bottom of the tube and serum separated. Percent of the packed cells were calculated to obtain final value. PCV was expressed in percentage (%).

3.12.1.3. Plasma Iron (PI)

Plasma iron was (PI) was estimated by Bathophenanthroline method as recommended by International Committee for Standardization in Haematology (ICSH, 1971). Briefly, 0.05 ml of plasma was added to 0.450 ml of the protein precipitant solution (aqueous solution made to contain 100g trichloroacetic acid, 30 ml thioglycolic acid and 2ml HCl per litre), mixed thoroughly and allowed to stand for 5 min and centrifuged at 2000rpm for 15 min to get an optically clear supernatant solution. Clear supernatant was transferred to a clean tube; equal amount of the chromogen solution (sodium acetate (2M) containing 250 mg bathophenanthroline disulfonate per litre) was added, mixed and allowed to stand for at least 5 min. The absorbance of the color developed was measured in a spectrophotometer at 535 nm. Iron standard was prepared by dissolving 70.2 mg of ferrous ammonium sulphate in water containing 0.2ml of 2N H₂SO₄ and made up to 1l. The iron concentration of the stock solution was 1 mg /100ml and final working standard concentration of 400 μg /100ml was made by diluting the stock and calibration curve was drawn. A regent blank was prepared by substituting water for plasma. Plasma iron was expressed as μg /100ml of plasma.

3.12.1.4. Total Iron Binding Capacity (TIBC)

TIBC was estimated by following the method mentioned in ICSH, (1978).To estimate TIBC, transferrin was saturated 100% by adding iron out side in Fe³⁺ form. For saturation 0.2 ml of 5 μg Fe /ml solution of FeCl₃ (This gives 10μg iron to each 1 ml serum, while unsaturated iron binding capacity of the serum ranges from 0-5μg/ ml in normal and pathological states) was added to 0.1ml of plasma and mixture was allowed to stand for 5 minutes, then 0.25% MgCO₃ was added to this solution and the tube agitated frequently and thoroughly for 15 min. After the final incubation, solution was centrifuged at 3000 rpm for 10 min. and supernatant was used for the
estimation. After chelating the iron not bound to transferring was estimated as in the case of plasma iron. TIBC was expressed as μg/100 ml of plasma.

3.12.1.5. Per cent saturation of transferrin (TSAT)
For determination of per cent saturation of transferrin, plasma iron was divided by TIBC and multiplied by the 100 to obtain final value. Values were expressed in percentage.

3.12.1.6. Unsaturated Iron Binding Capacity (UIBC)
UIBC was calculated as TIBC - PI and expressed as μg/100 ml of plasma.

3.12.1.7. Ferritin
Plasma ferritin was estimated by solid phase ELISA. Briefly, 20 μl of standard (ferritin diluted in human serum) or test plasma was dispensed into assigned wells of the microtitre plate followed by addition of 100 μl of Anti-Ferritin HRP Conjugate into each well. After mixing the contents thoroughly for 30 seconds, the plate was incubated for 45 minutes at room temperature. After incubation, the contents of the well were discarded and the plate was washed 5 times with distilled water. 100μl of substrate solution (3,3’,5,5’ Tetramethyl Benzidine in a citrate buffer) was added to each well, the plate was then gently shaken for 5 seconds and then incubated in dark for 20 minutes at room temperature. After 20 minutes, 100μl of stop solution (Hydrochloric acid diluted in purified water) was added to each well and gently shaken for 30 seconds. The absorbance was then taken immediately using an ELISA reader with 450 nm filter. Concentrations of test samples were determined by plotting a standard curve and the values were expressed in ng/ml.

3.12.2. Estimation of protein content
Protein concentrations were determined by the method as described by Lowry et al., (1951), using bovine serum albumin (BSA) as a standard. Lowry reagent (alkaline copper sulphate) was prepared by mixing Solution A containing 2% w/v sodium potassium tartarate (Rochelle salt), Solution B containing 1% w/v copper sulphate and Solution C containing 2% w/v anhydrous sodium carbonate dissolved in 0.1 N NaOH in the ratio, Solution A: Solution B: Solution C::1:1:98 freshly mixed just before estimation. The estimation was carried out in blood plasma of the test samples. 5 ml
of the freshly prepared Lowry reagent was added to the 10 µl of plasma followed by thorough mixing. The solution was allowed to stand for 30 minutes. After 30 minutes, 0.5 ml of the Folin-Ciocalteu reagent was added to the solution followed by vortexing. The color was allowed to develop and absorbance was taken after 30 min at 625 nm. For this, bovine serum albumin was used as the standard. Protein values were reported in mg/ml.

3.12.3. Assessment of Oxidative Stress

3.12.3.1. Malondialdehyde (MDA)
MDA as an index of lipid peroxidation was estimated in hemolysate according to the method of Niehaus & Samuelsol, (1968). 0.3 ml of hemolysate was mixed with 0.7 ml of 0.1 M phosphate buffer pH 7.4, and 2ml of TBA-TCA-HCl reagent containing 15% (w/v) TCA, 0.375 % (w/v) TBA and 0.25N HCl in the ratio of 1:1:1, incubated in boiling water bath for 30 minutes and centrifuged to obtain clear supernatant. Absorbance was measured at 534nm against the blank in which hemolysate was substituted by phosphate buffer solution. MDA was expressed as nmols of MDA per g of hemoglobin.

3.12.3.2. Superoxide dismutase (SOD)
Superoxide dismutase (SOD) was estimated by the modified method of Marklund and Marklund, (1974), which utilizes the inhibition of auto-oxidation of pyrogallol by superoxide dismutase. 0.05 ml of hemolysate was incubated with 0.05 M Tris succinate buffer (pH 8.5) at 37ºC and reaction started by adding 0.1 ml of 20mM pyrogallol .The increase in absorbance was recorded at 420 nm. The absorbance per minute was recorded. An appropriate blank was run simultaneously. SOD activity was expressed as units per gram hemoglobin, where a unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol auto oxidation. Copper-Zn SOD was estimated by this method. Its activity was expressed as units/gHb.

3.12.3.3. Ferric Reducing Ability of Plasma (FRAP)
FRAP was measured by using FRAP assay by Benzie and Strain, (1996). 40µl plasma was added to 2 ml of working FRAP solution containing acetate buffer, pH 3.6,
10mM; 2, 4, 6-tripyridyl-s-triazine (TPTZ) in 40 mM HCl and 20 mM FeCl₃.6H₂O, in the ratio of 10:1:1, at 37°C. Fe³⁺-TPTZ complex thus formed was measured against working FRAP at 593 nm through time scanning at 30 second intervals for 4 minutes. Increase in absorbance per minute was calculated for test plasma. The standard curve for 40 - 400μl FeSO₄ was also obtained. Quantification was done by regression analysis and the FRAP values were reported as μmol Fe (II)/ml of the plasma.

3.12.3.4. Glutathione peroxidase (GPx)

GPx activity was measured by Rotruck et al., (1973). 20 μl of plasma was added to reaction mixture containing 500 μl of 0.02% GSH, 100 μl of 10 mM NaN₃, 100 μl of 10mM H₂O₂, 380 μl of distilled water and 400 μl of 0.1 M Tris-Cl buffer (pH 7.4) and incubated at 37°C for 15 minutes. After incubation, 0.3 ml of 10% TCA was added to stop the reaction and the tubes were centrifuged to obtain clear supernatant. To this supernatant, 2 ml of 0.3 M Na₂HPO₄ and 0.1ml of Ellman’s reagent (19.5 g 5, 5’-dithiobisnitrobenzoic acid (DTNB) in 100ml 0.1% sodium citrate) was added. Standard curve for GSH was obtained simultaneously and absorbance of standard and test samples was read at 412 nm. The activity was expressed as μmol of GSH consumed/min/ml of plasma.

3.12.3.5. Uric acid (UA)

Plasma uric acid was measured by phosphotungstic acid method as given by Seligson, (1963). For standard curve, 1 to 5 ml of working standard (10μg/ml) was taken and raised the final volume up to 5 ml with water, then 1 ml of 10 % Na₂CO₃ added to this solution and allowed to stand for 10 min.1 ml of Phosphotungstic acid was added to it and allowed to stand for 30 min and reading were taken within 20 min. For sample, 0.5 ml of plasma was added to 4.5 ml of protein precipitant solution (50 ml of 10 % sodium Tungstate, 0.05ml of 85% orthophosphoric acid and 50 ml of 0.6 N H₂SO₄ was added to 800 ml of water, mixed thoroughly and stored it in dark bottle). Final volume of filtrate was made to 5 ml. 1ml of 10% NaCO₃ and 1ml of phosphotungstic acid reagent was added. Absorbance of Standard uric acid solution and samples was measured at 700nm.
3.13. In-vitro experiment

Many aspects of the toxicology of chemicals can be studied to a high degree of detail in in-vitro experiments. Information derived from in-vitro and other non-animal experiments is relevant only if the results are interpreted in the context of the in vivo situation (Blaauboer, 1996). With regard to heavy metals that are toxic and produced in various industries and people are exposed to them, this in-vitro studies provide society with the information needed to handle them in safe way. In-vitro studies from the basis of risk evaluation especially in case of toxic heavy metals. Advantage of in-vitro work is that it permits an enormous level of simplification of the system under study, so that the investigator can focus on a small number of components. By in-vitro studies one can incorporate more “science” in the “art” of toxicology (Nel et al., 2006).

In-vitro experiment was performed to corroborate the findings of in-vivo experiment. Among the metals studied in soil, water, food crops and blood in-vivo, Cd and Pb were the two most toxic metals and prominent in case of causing risk. So in-vitro experiment was carried out with the salts of Pb and Cd i.e. Pb(NO$_3$)$_2$ and CdCl$_2$.

### 3.13.1. Selection of respondents on basis of inclusion and exclusion criteria

Blood samples from respondents from control and coal mining exposed were collected for in-vitro experiments. These respondents were initially having the difference of exposure to mining activity. Inclusion and exclusion criteria were the same as in section 3.9.1 of this chapter. The objective of the study was explained to them and informed consent was obtained from them. A questionnaire developed for the purpose was filled, as far as possible. Their weight and height were measured. Blood of all participants was collected by trained technicians for biochemical analysis. Hb, Hct, BMI was assessed as given in section 3.12.1.1, 3.12.1.2 and 3.9 of this chapter.

The protocol of the study was approved by the Institutional Ethics Committee (IEC) of Population Resource & Research Centre (PRRC), Allahabad and informed consent to participate in the study was obtained from all participants.
3.13.2. Preparation of Pb(NO$_3$)$_2$ and CdCl$_2$ solutions

Pb(NO$_3$)$_2$ having molecular weight 331.2 g/mol was used to prepare 0.1 to 2 µM (0.1, 0.5, 1.0 and 2.0 µM) solution to cover the permissible level given by CDC, (2005), i.e. 10 µg/dl; to cover the range of exposure as given by WHO (Moyer et al., 2006) i.e. 30 µg/dl and a higher range (1 µM and 2 µM) which is toxic and is expected to show harmful effect.

CdCl$_2$ having molecular weight 183.317 g/mol was used to prepare 0.05 to 2 µM (0.05, 0.5, 1.0 and 2.0 µM) solution to cover the permissible range in blood given by WHO, (1996), i.e. 0.3-1.2 µg/l and also to cover the toxicological range to observe the harmful effects. 0.9 per cent saline was prepared for incubation of erythrocyte at 0 µM concentration that served as control against the experimental tubes of erythrocytes incubated with Pb and Cd.

3.13.3. Processing of erythrocytes with Pb(NO$_3$)$_2$ and CdCl$_2$ solutions

Blood samples in tubes with heparin were taken (with informed consent) from healthy male donors. Processing of blood samples was done as described in section 3.10 of this chapter. Erythrocytes were separated and washed with 0.9 per cent saline solution. Washed-packed erythrocytes were used in all analysis. For experimental manipulations, the packed cells were exposed to Pb (0.1, 0.5, 1.0 and 2.0 µM corresponding to 3-60 µg/dl of toxicological significance; for each control and coal mining exposed group) and Cd (0.05, 0.5, 1.0 and 2.0 µM corresponding to 0.9-360 µg/l of toxicological significance; the same samples were incubated with Cd too) incubating at 37 °C for 4 hours (Shafiq-ur-Rehman, 2013). For each sample of control and coal mining exposed group and for each treatment i.e. Pb and Cd packed RBCs were incubated with 0.9 per cent NaCl (saline) which served as 0 µM treatment. After 4 hours of treatment, erythrocytes were washed twice with phosphate buffer (PBS) and then lysed, and 1:20 hemolysates were prepared. Hemolysates were stored in -70°C until analysis. Flow diagram of in-vitro treatment is given below:
Figure 3.6. Flow chart of processing of blood and incubation for in-vitro experiment.

3.13.4. Estimation of OS markers Malondialdehyde (MDA) and Superoxide dismutase (SOD)
MDA as an index of lipid peroxidation was measured according to the method of Niehaus & Samuelsol, (1968) as mentioned in section 3.12.3.1 of this chapter. Superoxide dismutase (SOD) was quantified by the modified method of Marklund and Marklund, (1974), activity was expressed as units/g Hb as given in section 3.12.3.2 of this chapter.

3.14. Statistical analysis
The data were analyzed using Origin 6.1 and Microsoft Excel. Figures were made by Origin 6.1. Means and standard deviations were computed for all samples. Comparisons made by paired and unpaired t-tests were assessed and the minimum level of statistical significance was set at p < 0.05. Correlations between various estimations performed on the sample and between related groups were calculated using pearson’s correlation coefficients and expressed as r, the significance were determined using appropriate tests.
3.15. Materials

3.15.1. Chemicals

Chemicals with name, molecular formula, molecular weight and the manufacturer required for the study are listed in table 3.1.

Table 3.1. List of Chemicals.

<table>
<thead>
<tr>
<th>Products</th>
<th>Molecular weight (g/mol)</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,5-Dithiobis (2-nitrobenzoic acid) (C₁₄H₈N₂O₈S₂)</td>
<td>396.36</td>
<td>Himedia</td>
</tr>
<tr>
<td>Ellman’s reagent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4,6-Tri (2-pyridyl)-1,3,5-triazine (TPTZ)</td>
<td>312.34</td>
<td>Alfa Aesar</td>
</tr>
<tr>
<td>Acetic acid glacial 99-100% (A.R.grade)</td>
<td>60.05</td>
<td>Merck</td>
</tr>
<tr>
<td>Albumin Bovine Protease free fraction V powder (pH 6-7)</td>
<td></td>
<td>SRL</td>
</tr>
<tr>
<td>Ammonium iron (II) sulphate hexahydrate (G.R. grade) (NH₄)₂Fe(SO₄)₂.6H₂O</td>
<td>392.14</td>
<td>Merck</td>
</tr>
<tr>
<td>Ammonium molybdate (NH₄)₆Mo₇O₂₄.H₂O</td>
<td>1235.9</td>
<td>Fisher scientific</td>
</tr>
<tr>
<td>Bathophenanthroline disulphonate disodium salt extra pure (A.R. grade)</td>
<td>536.48</td>
<td>SRL</td>
</tr>
<tr>
<td>C₂₄H₁₄N₂Na₂O₆S₂</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cadmium chloride (CdCl₂)</td>
<td>183.31</td>
<td>Merck</td>
</tr>
<tr>
<td>Calcium carbonate (CaCO₃)</td>
<td>100.09</td>
<td>Merck</td>
</tr>
<tr>
<td>Copper sulphate (CuSO₄.5H₂O) (A.R.grade)</td>
<td>159.60</td>
<td>Merck</td>
</tr>
<tr>
<td>Cyanmethemoglobin standard</td>
<td></td>
<td>Span diagnostics Ltd.</td>
</tr>
<tr>
<td>Di potassium hydrogen phosphate (K₂HPO₄)</td>
<td>174.18</td>
<td>Merck</td>
</tr>
<tr>
<td>Di sodium hydrogen phosphate (Na₂HPO₄), (A.R.grade)</td>
<td>141.95</td>
<td>Merck</td>
</tr>
<tr>
<td>Drabkin’s solution (Diluent)</td>
<td></td>
<td>Loba chemie</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA) [CH₂.N(CH₂.COOH)CH₂.COONa]₂.2H₂O</td>
<td>372.24</td>
<td>Fisher scientific</td>
</tr>
<tr>
<td>Ferric chloride (FeCl₃) (A.R.grade)</td>
<td>162.2</td>
<td>Merck</td>
</tr>
<tr>
<td>Ferrous sulphate (FeSO₄) (A.R.grade)</td>
<td>278.02</td>
<td>Merck</td>
</tr>
<tr>
<td>Material</td>
<td>Supplier</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>Folin-Ciocalteu’s phenol reagent</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Glutathione (Reduced) 99% (C_{10}H_{17}N_{3}O_{6}S)</td>
<td>Loba cheme</td>
<td></td>
</tr>
<tr>
<td>Hydrochloric acid (HCl) 35% pure (A.R.grade)</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Hydrogen peroxide 30% purified (H_{2}O_{2})</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Lead nitrate Pb(NO_{3})_{2}</td>
<td>Qualigen fine chemicals</td>
<td></td>
</tr>
<tr>
<td>Lithium carbonate (G.R. grade) (Li_{2}CO_{3})</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Lithium sulphate monohydrate (Li_{2}SO_{4}.H_{2}O)</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Magnesium carbonate (MgCO_{3}) (A.R.grade)</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Na-K-Tartarate 4H_{2}O</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Nitric acid (HNO_{3}) about 70%</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Orthophosphoric acid (H_{3}PO_{4})</td>
<td>Loba</td>
<td></td>
</tr>
<tr>
<td>Pathozyme Ferritin Ref OD407. ELISA kit</td>
<td>Omega diagnostics</td>
<td></td>
</tr>
<tr>
<td>Perchloric acid about 70% (HClO_{4})</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Phosphotungstic acid (H_{3}PW_{12}O_{40}), (A.R.grade)</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Potassium dihydrogen phosphate purified (KH_{2}PO_{4})</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Pyrogallol (1,2,3-trihydroxybenzene), (C_{6}H_{6}O_{3})</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Sodium acetate anhydrous (CH_{3}COONa)</td>
<td>SRL</td>
<td></td>
</tr>
<tr>
<td>Sodium azide (NaN_{3}), (A.R.grade)</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Sodium carbonate (Na_{2}CO_{3}), (A.R.grade)</td>
<td>Fisher scientific</td>
<td></td>
</tr>
<tr>
<td>Sodium dihydrogen phosphate dehydrate crystal pure (NaH_{2}PO_{4}.2H_{2}O)</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Sodium disulfite purified (Na_{2}S_{2}O_{3})</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Sodium Hydroxide (NaOH), (A.R. Grade)</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Sodium sulphite anhydrous purified. (Na_{2}SO_{3})</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Sodium tungstate dihydrate purified (Na_{2}WO_{4}.2H_{2}O)</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>ß mercepto ethanol (BME), (A.R.grade)</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Succinic acid (G.R. grade) (C_{4}H_{6}O_{4})</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Sulphuric acid (H_{2}SO_{4}) about 98%</td>
<td>Merck</td>
<td></td>
</tr>
<tr>
<td>Thiobarbituric acid (TBA) (G.R.grade) (C_{1}H_{4}N_{2}O_{2}S)</td>
<td>Loba cheme</td>
<td></td>
</tr>
</tbody>
</table>
### Materials & Methods

**Thioglycolic acid 80% (HSCH$_2$COOH)**
- 92.12
- Loba chemie

**Trichloro acetic acid (TCA), (A.R) (CCl$_3$COOH)**
- 163.39
- Merck

**Tris buffer (C$_4$H$_{11}$NO$_3$) (G.R.grade)**
- 121.14
- Merck

**Uric acid (C$_5$H$_4$N$_4$O$_3$)**
- 168.11
- Titan biotech

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#### 3.15.2. Glass ware

All glass ware were obtained from Borosil, India. Washed thoroughly with labolene and chromic acid and then with tap water and rinsed with distilled water. The glass wares were dried in hot air oven. Consumables such as microtips, eppendorf tubes, reagent bottles etc. were obtained from Tarson, India. ACD and EDTA vials were obtained from Poly Medicure Ltd., India.