

EXPERIMENTAL PROCEDURE

Silver is a naturally occurring metal that is widely utilized in photographic industry, jewellery industry, electronics and electrical applications (Purcell and Peters, 1998). Industries are the major source of silver pollution particularly jewellery industry which utilize higher concentration of silver for gold fabrication. Hence the present study was aimed to assess the health status of jewellery unit workers.

It was observed by Figueira *et al.* (2012) that metallothioneins (MTs) are excellent biomarkers against metal stress. Among the most used biomarkers for pollution in the environment, MTs have been particularly useful as a monitoring device or a biochemical indicator of metal exposure (Chaffai *et al.*, 2000). Several researchers proved that MTs can protect organisms from metal toxicity due to their binding ability to metals and also MTs expression get raised with the increase of metal concentration in tissue (Viarengo *et al.*, 2000). Based on the above facts, *in vivo* and *in vitro* studies were conducted in peripheral blood lymphocytes to find out the relationship between the silver and metallothioneins, which probably might help to understand the role of MTs in metal stress.

The experimental design adopted in the study “***In vivo* and *In vitro* Studies on the Expression of Metallothioneins in Response to Silver Exposure among Jewellery Unit Workers**” was conducted in three phases and it is discussed under the following headings:

PHASE I

3.1 Assessment of the Health Status of Workers in Jewellery Units

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- 3.1.11 Assessment of biochemical parameters of renal function
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- 3.3.4 Analysis of uptake of AgNO_3 by cultured lymphocytes using Transmission Electron Microscope (TEM)
- 3.3.5 Isolation of metallothioneins from cultured lymphocytes
- 3.3.6 Free radical scavenging activity of metallothioneins
- 3.3.7 Quantification and identification of functional groups of metallothioneins from cultured lymphocytes

3.4 Statistical Analysis

PHASE I

3.1 Assessment of the Health Status of Workers in Jewellery Units

A cross sectional study was conducted on workers engaged in manufacturing precious metals. Alloys and chemicals are used for melting, refining, welding and polishing the jewellery. These substances are potentially harmful to human health. Liver and kidney are important organs of detoxification, metabolism and storage of important metabolites and excretion of xenobiotics. Hence the present study was aimed to assess the liver and kidney functions of the jewellery unit workers.

3.1.1 Study area and study population

Coimbatore is one of the fastest growing second-tier cities in India and a major industrial, commercial, educational, information technology, healthcare and manufacturing hub of Tamil Nadu. It is one of the major gold jewellery manufacturing centers in India, renowned for making cast jewellery and machine made jewellery. The city is home to about 3000 jewellery manufacturing companies and to over 40,000 goldsmiths. The city was selected for the study because of nearness of the jewellery units to the investigator.

Workers of these units are exposed to fumes and dusts which consist of metals and hazardous compounds. Many of these substances are potentially harmful to human health. Workers in these units inhale and absorb large amounts of these substances daily over extended periods of time and exposure is principally through inhalation, ingestion and dermal absorption. The jewellery workers live and work in small rooms which do not have adequate ventilation. These workers do not seem to wear protective masks, shoes, personal protective clothes and gloves.

Plate 1 shows the different processes carried out in the jewellery units and the work environment of the selected workers

A cross sectional study was conducted in the jewellery making units located in Coimbatore (Kuniamuthoor, Saibaba Colony and R.S Puram) Tamil Nadu. Cross-sectional study is a type of observational study that involves data collection from a population or a representative subset, at one specific point of time.

Plate 1
Jewellery Manufacturing Process

Twenty Four Karat Bullion



Melting

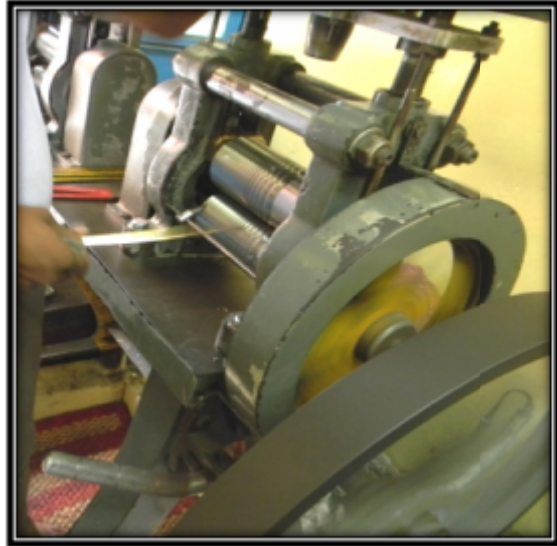


Molding



Plate 1 (contd...)
Jewellery Manufacturing Process

Making of Gold Thinner Plates



Making of Gold Wire and Pipes

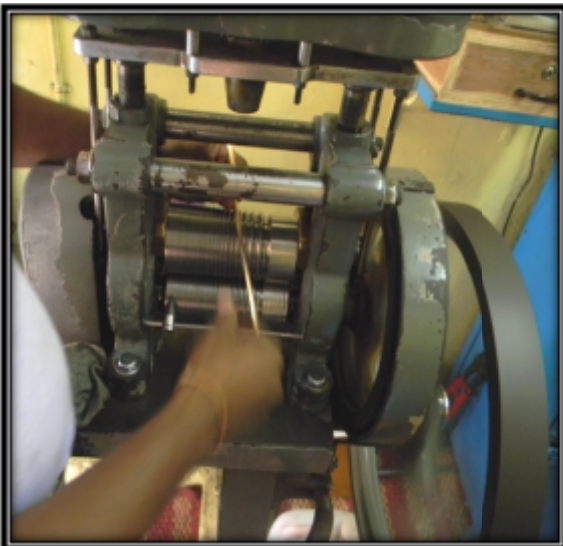


Plate 1 (contd...)
Jewellery Manufacturing Process

Shouldering



Polishing



Finishing



Jewellery unit workers were designated as exposed group. Age and sex matched individuals who are living in the same area and not working in these jewellery units served as controls.

Blood and urine samples were collected from both group workers. Male workers were selected for the study as female workers in this occupation were very less. The study protocol was approved by the Human Ethical Committee (HEC.2011.27) of Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, Tamil Nadu.

3.1.2 Administration of questionnaire and informed consent form

Each study subject received a questionnaire and was orally explained in local language about name, age, employment, anthropometric measurements, residences and food habits as well as medical history, especially regarding diabetics, kidney and liver diseases. An informed written consent was obtained from control and jewellery unit participants before the data and sample collection (Appendix 1).

The study was designed with selected inclusion and exclusion criteria for the selection of the participants

Inclusion criteria:

Exposed groups : Workers with minimum one year of experience in these units

Control groups : Workers with no relation to making of jewellery

Male workers in the age group of 20-50 years

Exclusion criteria:

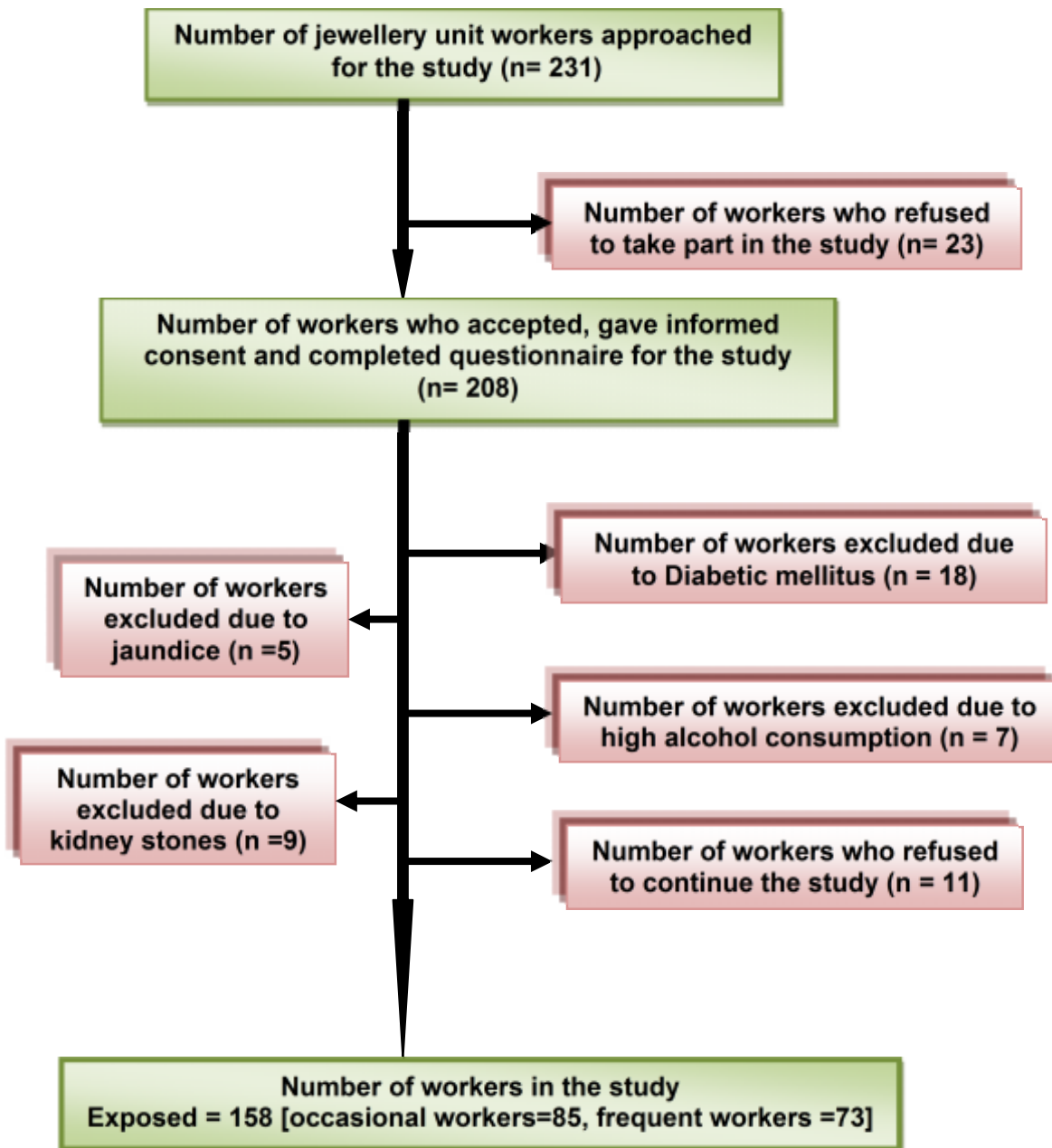
Workers with known history of diabetes mellitus, kidney and liver diseases, blood transfusion and any other pathological condition

A prospective study observes for outcomes, such as the development of a disease, during the study period and relates this to other factors such as suspected risk or protection factors. Workers with known history of diabetes mellitus, kidney and liver diseases, blood transfusion and any other pathological condition were excluded from the study because these known factors may obstruct the findings of confounding factors

or outcome of the results. Workers who were less than twenty years were not included due to ethical reasons and also were poorly represented in the selected work places.

Figure 6

Selection of Jewellery Unit Workers for the Study



3.1.3 Grouping of jewellery unit workers

Based on the information collected from the workers, jewellery unit workers (exposed group) were further categorized into two groups as occasional (Group B) and frequent workers (Group C). Controls were denoted as group A. Frequent workers included those working in jewellery units for a significant time period for a particular wage. Occasional workers are those have been working by booking order for jewellery items which happens at irregular or infrequent intervals.

3.1.4 Collection of demographic data

Demographics are the quantifiable statistics of a population. It includes age, ethnicity, education, general health details and professional status.

Cigarettes carry serious health risks, which are more prevalent than in other tobacco products (Zhu *et al.*, 2007). The resulting use of tobacco is the single greatest cause of preventable death globally (World Health Organization, 2008). Smoking may lead to diseases which most commonly affects the heart, liver and lungs. It is a major risk factor for heart attacks, strokes, chronic obstructive pulmonary disease (COPD) including emphysema and chronic bronchitis. Cigarette smoking may be a factor that contributes to the occurrence of cancer particularly in lung, mouth, pancreatic and liver. It also causes peripheral vascular disease and hypertension (Centers for Disease Control and Prevention, 2007).

Alcohol consumption also causes major health problems, including cirrhosis of the liver, depression, seizures, gout, high blood pressure, nerve damage, anemia and cardiovascular disease. These two factors may influence the result of the present study. Hence in the present study smoking and history of alcohol consumption were recorded among the workers.

Smoking habits of the selected participants (current smokers) was expressed in pack years which is calculated in terms of number of pack years = (number of cigarettes smoked per day × number of years smoked) /20; 20= number of cigarette in a pack. Alcohol consumption of the selected participants was stated in units/week (1 unit = 10 ml).

3.1.5 Anthropometric measurements

It is the measurement of size and proportion of the human body such as height and weight. Height was measured by using a steel measuring scale fixed to the wall with minimum height of 0.1 cm. Weight was measured using a weight scale ranging from zero up to a maximum of 180 kg. It has been found that BMI (body mass index) is the best alternative for body fat percentage among ratios of weight and height. It is calculated using the formula

$$\text{BMI} = \frac{\text{Weight in kg}}{(\text{Height in meter})^2} \quad (\text{Oldenburg } et al., 2013)$$

Blood pressure is the measure of force of blood pushing against blood vessel walls. The exact causes of high blood pressure are not known, but several factors and conditions may play important role in its development includes smoking, obese, lack of physical activity, alcohol consumption, stress, older age and genetics. Occupational setting is also one of the factors for high blood pressure of an individual. Hence in the present study, systolic and diastolic blood pressure were measured in selected population using Omron Blood Pressure Monitor: HEM 7112 which was expressed in mm Hg.

3.1.6 Collection of blood and urine samples

Peripheral venous blood samples (5 ml) were collected from the subjects. All samples for serum (Vacutainers - AcCuvet-PLUS with clot activator) were centrifuged at 3000 rpm at 4°C for 10 minutes. The samples were aliquoted into labeled cryovials and stored at - 20°C for further analysis. Whole blood samples were used for hematological analysis. Fresh blood samples were preserved in ice bags during the collection of samples. Workers who have high levels of serum silver and metalloproteins in the exposed groups (n = 3) and controls (n = 3) were selected based on their consent. Ten ml of blood samples were collected for second time. Ten ml of peripheral venous blood samples were collected from willing healthy donors of control group (n = 3) for cell

cultures study for second time. Spot urine samples were collected from subjects in sterile 50 ml polypropylene containers. Urine samples were preserved in ice bags during the collection of samples and aliquoted into labeled cryovials and stored at - 20°C until analysis.

3.1.7 Assessment of hematological indices

Complete blood count (CBC) is a test panel that gives information about the cells in blood (Buttarelo and Plebani, 2008). The cells that circulate in the bloodstream are generally divided into three types such as white blood cells (leukocytes), red blood cells (erythrocytes) and platelets (thrombocytes). Abnormally high or low counts may indicate the presence of many forms of disease and hence blood counts are amongst the most commonly performed blood tests in medicine, as they can provide an overview of general health status of an individual (Blesi *et al.*, 2011).

CBC was analyzed in the whole blood of the participants in three part hematological analyzer (Sysmax KX-21). It includes Total Leucocytes and Differential Count, Red Blood Count, Hemoglobin Concentration, Hematocrit (HCT), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC), Platelet Count, Mean Platelet Volume (MPV), Platelet Distribution Width (PDW) and Red Blood Cell Distribution Width (RDW). The detailed procedure for CBC by Sysmax KX-21 is given in Appendix 2.

3.1.8 Estimation of serum glucose

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Chronic diabetes is associated with long-term damage, dysfunction and failure of various organs, especially the eyes, kidneys, nerves, heart and blood vessels (American Diabetes Association, 2008). Estimation of glucose in blood is an important parameter assessed in the workers to rule out the DM from the study. Random blood glucose was analyzed by using kit (Agappe diagnostics) based on GOD-PAP method (Appendix 3).

3.1.9 Assessment of oxidative stress

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and the levels of antioxidant defense system. In humans, oxidative stress is thought to be involved in the development of cancer, Parkinson's disease, atherosclerosis, heart failure and myocardial infarction (Devasagayam, 2004). The oxidative damage is tested by the increase in lipid peroxidation formation.

Among the cellular molecules, lipids contain more than one double bond susceptible to action of free radicals. The resulting reaction is known as lipid peroxidation which produces a large number of by products being studied in relation to diseases. These by products can be measured by the estimation of aldehyde products using thiobarbituric acid (Devasagayam, 2003). Heavy metals induce an increased production of malondialdehyde (MDA), an indicator of lipid peroxidation and whole body free radical activity (Flora *et al.*, 2008). High levels of lipid peroxides are associated with cancer, heart disease, stroke and aging. The details of analysis of this biomarker are elaborated in Appendix 4.

3.1.10 Assessment of biochemical markers of liver function

Diagnostic laboratories assess the functions of the organs through various biochemical parameters. Biochemical analysis in the jewellery unit workers might help to identify incidence of metal toxicity in this group of the study population.

Liver regulates and controls many important metabolic function. It is of interest to find the level of hepatic damage with the load of heavy metals. Liver function tests (LFTs) are a group of blood tests that detect inflammation and damage to the liver. It includes Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Total protein, Albumin, Cholesterol and Bilirubin. The methods of analysis, the reference details for the methodology and the appendices in which they are elaborated are given in Table1.

Table 1

Details of the Liver Function Markers Estimated in the Serum Sample of Jewellery Unit Workers

Parameter	Method	Reference	Appendix
Aspartate aminotransferase	Spectrophotometry-DNPH method	Reitman and Frankel, 1957	5
Alanine aminotransferase	Spectrophotometry-DNPH method	Reitman and Frankel, 1957	6
Alkaline phosphatase	Spectrophotometry-Kinetic method	Tietz, 1983	7
Albumin	Spectrophotometry-BCG Dye Method	Doumas, 1971	8
Total protein	Spectrophotometry-Biuret method	Young <i>et al.</i> , 1975	9
Total and direct bilirubin	Spectrophotometry-Diazo method	Pearlman and Lee, 1974	10
Cholesterol	Spectrophotometry-CHOD-PAP method	Roeschlau <i>et al.</i> , 1974	11

AST/ALT ratio is used to differentiate between causes of liver damage or hepatotoxicity (Gopal and Rosen, 2000). Hence in the present study AST/ALT ratio was calculated to know hepatic damage of the selected population.

3.1.11 Assessment of biochemical parameters of renal function

The kidneys contain many different cell types with their primary roles concerned with glomerular filtration, tubular reabsorption and concentration of metabolites (Stevens *et al.*, 2006; Al-Gehani, 2013). The present study was performed to assess the function of the kidney in the chosen population. As biochemical parameters of the kidney function, creatinine, uric acid and urea concentration in serum as well as random urinary protein and creatinine levels were determined. Total protein to Creatinine Ratio (P/C) was calculated.

The details of the assay procedures, references and appendices for renal function indicators are given in Table 2.

Table 2
Details of the Renal Function Indicators Estimated in the Serum and Urine Samples of Jewellery Unit Workers

Parameter	Method	Reference	Appendix
Serum urea	Spectrophotometry-GLDH- urease method	Young,1990	12
Serum uric acid	Spectrophotometry-Uricase method	Barham and Trinder,1972	13
Serum creatinine	Spectrophotometry-Jaffe's method	Bartels <i>et al.</i> 1972	14
Urine total protein	Spectrophotometry-Bradford Method	Bradford, 1976	15
Urine creatinine	Spectrophotometry-Jaffe's method	Toro and Ackermann, 1975	16

Normal individuals usually excrete very small amounts of protein in the urine. Persistently increased protein excretion is usually a marker of kidney damage. Quantifying protein in urine is commonly used in the diagnosis of kidney diseases. The protein: creatinine ratio of an untimed urine could be used as a reliable indicator of the 24 hours protein excretion (Price *et al.*, 2005; Xin *et al.*, 2004; Fulks *et al.*, 2012). According to the National Kidney Foundation (NKF) and clinical practice guidelines under most circumstances untimed spot urine samples can be used to detect and monitor proteinuria in children and adults (Chavan *et al.*, 2011).

$$\text{Protein to Creatinine ratio (P/C) (mg/mg)} = \frac{\text{Urine protein (mg/dl)}}{\text{Urine creatinine (mg/dl)}}$$

3.1.12 Analysis of silver and metalloprotein

Silver ions (Ag^+) strongly increase the production of reactive oxygen species and also induce the expression of metalloprotein such as metallothioneins (Krott *et al.*, 2009). Metallic silver is used as an alloy in gold manufacturing unit to produce a wide range of karat gold. Hence in the present study serum levels of silver ions and metallothioneins were investigated.

1. Silver

Pure gold is alloyed with copper, zinc, cadmium and silver in varying proportions to produce a wide range of karat gold. The level of serum silver was assessed by flame atomic absorption spectroscopy (SHIMADZU, AA- 7000).

Serum silver status might reflect the past and present, acute and chronic silver exposure among the workers. Silver is used in higher concentration in gold fabrication as an alloy than other metals and also it is a xenobiotic element with less recognized trace metal value in the human body. Thus the study was more concentrated on silver exposure.

2. Metallothioneins

Metallothioneins (MTs) are free radical scavengers which bind to a number of trace metals and also save cells and tissues from heavy metal toxicity. Expression of MT in cells, however, can protect against lipid peroxidation by scavenging free radicals (Hurnanen *et al.*, 1997). MTs can be used as biomarker in the field of industrial health due to the synthesis of MTs and the accumulation of metals in the cells and the tissues (Sakulsak *et al.*, 2009).

The assay details of silver and metallothioneins in serum in the jewellery unit workers are given in Table 3.

Table 3

Details of Estimation of Silver and Metallothioneins in the Serum Samples of Jewellery Unit Workers

Parameter	Method	Reference	Appendix
Silver	Atomic absorption spectrophotometry	Boosalis <i>et al.</i> , 1987	17
Metallothioneins	Spectrophotometry-Ellman's method	Viarengo <i>et al.</i> , 1997	18

3.1.13 Assessment of serum mineral status

Toxic metals occasionally imitate the action of an essential element in the body, interfering with the metabolic process to cause illness. Disturbances in calcium and phosphorus metabolism are almost invariable consequences of chronic kidney disease (CKD) (Goodman, 2005). Estimation of calcium and phosphorus in jewellery unit workers might help to evaluate the extent of metal exposure.

Toxic heavy metals are able to obstruct at both the levels by a variety of mechanisms. Mostly these metals hinder calcium metabolism in both kidneys and in bones (Nordberg *et al.*, 2011). Calcium is absorbed with phosphorus and is a synergist in bone formation (Nord, 2009). Since phosphorus metabolism is associated with calcium, estimation of serum phosphorus level might propose any damage due to silver metal. The details of analysis of these minerals in serum in the jewellery unit workers are given in Appendices 19 and 20.

PHASE II

3.2 Isolation, Molecular Mass Determination and Quantification of Metallothioneins from Peripheral Blood Lymphocytes of Selected Workers in the Jewellery Units

Metallothioneins (MTs) belong to low molecular mass, cysteine-rich proteins with a variety of functions including involvement in free radical scavenging, metal

homeostasis, protection against heavy metal damage and in metabolic regulations. The over expression of MTs in proliferating cells is of interest to the study of MTs as a biochemical marker for metal exposure. Levels of MTs in invertebrates and aquatic vertebrates are well associated with heavy metal pollution of an environment (Ryvolova *et al.*, 2011). Hence the study was designed to isolate, determine the molecular mass and quantify the MTs from peripheral blood lymphocytes of silver exposed groups which were compared with controls.

3.2.1 Isolation of metallothioneins from peripheral blood lymphocytes of the selected workers

Lymphocytes are small white blood cells and they are the only blood cells which can be transformed into actively proliferating cells. They help to protect the body from diseases, invasion of foreign bodies, tumors and infections. Separation of lymphocytes from whole blood using Ficoll Hipaque 1077 (Sigma-Aldrich) is based on the method of Boyum (1968). It is an aqueous solution of a high molecular weight polysaccharide and sodium diatrizoate, an iodinated nonionic compound, adjusted to a density of 1.077 ± 0.001 . This medium facilitates rapid recovery of viable lymphocytes from small volumes of blood. The participants who had high levels of metallothionein and silver content in serum were selected. The peripheral blood lymphocytes (PBLs) were isolated from fresh heparinised blood removed by vein puncture from selected exposed (n=3) and unexposed (n=3) donors. PBLs were isolated using Ficoll Hipaque 1077 density gradient from freshly collected venous blood (<http://www.Kluniversity.in/elearn/.../sqnaugaghj75393SQNAUGAGHJ.pdf>). The detailed procedure for isolation of lymphocytes is discussed in Appendix 21.

Metallothioneins are a valid biomarker for metal exposure. 1D/2D gel electrophoresis, spectrophotometric analyses and SEC-HPLC-ICP-MS are the methods to identify the presence of MT in the biological samples (Hauser-Davis *et al.*, 2012). Hence the present study was performed for the isolation, molecular mass determination and quantification of metallothionein by gel filtration, Sodium-Dodecyl Sulfate Poly Acrylamide Gel Electrophoresis (SDS PAGE) and High Performance Liquid Chromatography (HPLC) respectively.

Isolated lymphocytes were centrifuged at 2000 rpm for 10 minutes to obtain the pellets. Cell pellets were lysed in cooled lysing buffer for 15 minutes, in which to a final concentration of 1 µg/ml of AgNO₃ was added to prevent the oxidation of MTs. The cell debris was pelleted by centrifugation (15,000 g for 15 minutes at 4°C). The composition of cell lysing buffer is discussed in Appendix 22. The supernatant was heated at 70° C for 5 minutes and allowed to cool to room temperature and respun the lysate (15000 g for 15 minutes at 4° C). Gel filtration column was prepared in Sephadex G-75 (superfine) column (1 x 30 cm) and equilibrated with tris HCl buffer at pH8.6 (0.02 M Tris and 0.01 M NaCl). The supernatant was eluted on the column with the same buffer at a rate of 15 ml/h. The fraction volume was 1.5 ml. Detailed procedure of gel filtration is discussed in Appendix 23.

Protein identification was performed after elution in UV spectrophotometer by measuring the absorbance at A250 and A280. The ratio of absorbance at 250 nm/280 nm more than 1 indicating the presence of mercaptide bonds (Gulati *et al.*, 1987) was further confirmed by the presence of sulfhydryl groups of eluted protein. The presence of metallothioneins was confirmed by using metallothionein standard in HPLC analysis.

3.2.2 Estimation of sulfhydryl groups in the isolated metallothioneins

It is well known that when an organism is exposed to heavy metals it synthesizes low molecular mass thiol compounds rich in cysteine such as metallothioneins and glutathione. These compounds contain sulfhydryl groups (thiol) for binding of a variety of metals. Several studies have concluded that exposure to metals cause increase of thiol compounds in living body (Kovarova *et al.*, 2009). Recently, several studies on thiol containing compounds of *crustaceans* and in invertebrates were carried out for assessment of metal contamination (Amiard *et al.*, 2006; Won *et al.*, 2008). Hence in the present study the levels of thiol groups in isolated proteins from peripheral blood lymphocytes were estimated. Eluted fractions with the ratio of absorbance at 250 nm/280 nm more than 1 were pooled together and concentrated in freeze dryer (Christ, alpha-1-4 LD plus). Based on the sulfhydryl content of the proteins, it was assumed that it might be MTs and further verified by HPLC analysis. Their sulfhydryl content was assessed in the concentrated samples by Ellman method (Appendix 24).

3.2.3 Determination of molecular mass of isolated metallothioneins

Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) belongs to the most commonly and routinely used method for the determination of proteins (Aoki and Suzuki, 1991). The concentrated samples were heated in a water bath (95°C) for 3 minutes and loaded on SDS PAGE gel according to the modified method of Aoki and Suzuki, 1991 (Appendix 25). After electrophoresis, proteins were stained with silver stain by modified method of Merrill *et al.* (1988) discussed in Appendix 26.

3.2.4 Quantification of isolated metallothioneins

There are several in vitro studies carried out to identify the presence of metallothionein in liver and kidney tissues. But recent studies have proved that metal treated lymphocytes can be used for MT isolation by gel filtration and RP-HPLC. Automatic Edman-degradation of the different fractions revealed the presence of different isoforms of MTs (Vandeghinste *et al.*, 2000). In the present study MTs from concentrated proteins of PBLs were quantified by HPLC method using horse kidney metallothionein (Sigma) as reference standard. The elaborated procedure is discussed in Appendix 27.

3.2.5 Identification of functional groups of isolated metallothioneins

The present research was conducted to identify the functional groups of isolated proteins using FTIR (Fourier Transformed Infra Red-Shimadzu) method. The scanning process was conducted between wavenumber 400 and 4000 cm^{-1} . Infrared radiation is passed through a sample where it is absorbed and some of it is passed through the sample. The resulting spectrum represents the molecular absorption and transmission, creating a molecular fingerprint of the sample. The determination of functional group was done by comparing wavenumber of functional groups of the protein samples to existing standard (Shi *et al.*, 2002). The detailed procedure is discussed in Appendix 28.

PHASE III

3.3 Evaluation of Metallothioneins in the Cultured Lymphocytes of the Selected Workers on Exposure to Silver ions

Metallothioneins (MTs) are low molecular weight proteins which bind to a number of heavy metals through their cysteine residues. They are believed to be involved in essential metal metabolism and in heavy metal detoxification. Several studies revealed that human peripheral blood lymphocytes have the capacity to synthesize MTs in response to several heavy metals (Jonai *et al.*, 1992). This study was aimed to monitor the heavy metal exposure of individual workers by estimating their MT levels of lymphocytes through cell culture method. MT levels were assessed in human peripheral blood lymphocytes exposed to various concentrations of silver nitrate (AgNO_3) *in vitro*.

3.3.1 Cell culture

Ten ml of peripheral blood was taken from each of three healthy donors and lymphocytes were isolated by centrifugation of the blood samples through Ficoll-paque-1077 step gradients as described in phase II study (Appendix 21). The isolated lymphocytes were washed twice in phosphate buffer saline (PBS) and once in RPMI 1640 medium. Isolated PBLs were then divided into 5 aliquots and incubated in cultured flasks (Duron schott bottle Borosil, 250 ml) containing RPMI 1640 supplemented with 20% heat-inactivated fetal bovine serum, 40 $\mu\text{g}/\text{ml}$ of phytohemagglutinin-M (PHA-M), 62.5 U of penicillin/ml and 62.5 μg of streptomycin/ml. The cells were incubated at 37° C under 5% CO_2 atmosphere. PHA-M is a mitogen which stimulates lymphocytes proliferation. After 24 hours of incubation, the aliquots of lymphocytes were treated with silver nitrate (AgNO_3) in varying concentration namely 0.5 μM , 1 μM , 2 μM and 4 μM and incubated in varying time periods namely 3 hours, 6 hours, 12 hours, 24 hours and 48 hours. The same volume of PBS was added to another aliquot as control. At each time point, the cells were harvested (centrifuged for 10 minutes at 5000 rpm) and washed in fresh RPMI prior to analysis of cell cytotoxicity and cell viability was analyzed after 24 hours.

3.3.2 Determination of cell viability

The cell viability or dye exclusion test is applied to find out the number of viable cells in the cell suspension. The living cells have intact cell membrane that exclude the dye such as trypan blue and nonviable cell have broken cell membrane that uptake the dye. Hence a viable cell will have a clear cytoplasm whereas a nonviable cell will have a

blue cytoplasm (Strober, 2001). The cell suspension is mixed with dye and examined under the microscope to determine whether cells take up or exclude dye. The number of stained cells and the total number of cells were counted in the cell suspension using hemocytometer. Different concentration of AgNO_3 (0.5 μM , 1 μM , 2 μM and 4 μM) exposed cells were harvested after 24 hours and viable cells were counted using hemocytometer by trinocular phase contrast microscope (Kozo Optics, Japan). The detailed procedure for dye exclusion test is given in Appendix 29.

3.3.3 Determination of cell cytotoxicity

The mitochondrial activity of living cells is determined by the conversion of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) into formazan crystals. For most cell population, the total mitochondrial activity is related to the number of viable cells and this assay is generally used to determine the *in vitro* cytotoxic effects of drugs on cell lines or primary patient cells (Meerlo *et al.*, 2011). The *in vitro* cytotoxic effects of chosen concentrations of AgNO_3 (0.5 μM , 1 μM , 2 μM and 4 μM) on lymphocytes at each time points (3 hours, 6 hours, 12 hours, 24 hours and 48 hours) selected for the study were measured. Cells were also exposed to a single dose of H_2O_2 (50 μM) for the same time points as an indicator of cytotoxicity from a chemical known to cause loss of cell viability by oxidative stress (positive control). PBS treated unexposed cells were used as control. MTT assay protocol is discussed in Appendix 30.

3.3.4 Analysis of uptake of AgNO_3 by cultured lymphocytes using Transmission Electron Microscope (TEM)

Transmission Electron Microscopy (TEM) yields information on the internal structure of materials. It utilizes energetic electrons to provide morphologic, compositional and crystallographic information on samples. TEMs are the most powerful microscopes which produce high-resolution, two-dimensional images, allowing for a wide range of educational, research and industrial applications.

AgNO_3 causes a dose and time dependent loss of cell viability in PBLs. Only 50% of cells remained viable in 4 μM dosage at 48 hours. Hence an attempt was made to analyze the internal changes happening in AgNO_3 exposed lymphocytes (4 μM) after 24

hours and compared with control lymphocytes. The detailed study protocol is given in Appendix 31.

3.3.5 Isolation of metallothioneins from cultured lymphocytes

The present study was aimed to isolate metallothioneins of cultured lymphocytes exposed to AgNO₃ at different concentrations namely 0.5µM, 1µM, 2µM and 4µM after 24 hours. The study protocol was followed as mentioned in Phase II study (Appendices 21, 22, 23 and 24).

3.3.6 Free radical scavenging activity of metallothioneins

One or more unpaired electrons are present in free radical molecules and make the molecules more reactive. The free radical molecules play a dual role in biological systems, since they can be either helpful or harmful for living systems. There are a number of mechanisms involved in the protection of cells against free radicals. Metallothioneins participate in an array of protective stress response (Nedecky *et al.*, 2013).

Metallothioneins and other sulfhydryl groups containing amino acids have the ability to scavenge superoxide radicals (Hussain *et al.*, 1995), hydrogen peroxide (Anderson *et al.*, 1999), ABTs radicals (Atif *et al.*, 2006), DPPH radicals (Ai *et al.*, 2013) and hydroxyl radicals (Ercal *et al.*, 2001) in dose dependent manner.

The present study was designed to find out the free radical scavenging activity of isolated metallothioneins from AgNO₃ exposed lymphocytes of different concentration (0.5µM, 1µM, 2µM and 4 µM) after 24 hours and compared with control. ABTS, DPPH, superoxide and hydrogen peroxide scavenging activity of metallothioneins assay procedure is discussed in Appendices 32, 33, 34 and 35 respectively.

3.3.7 Quantification and identification of functional groups of metallothioneins from cultured lymphocytes

High performance liquid chromatography (HPLC) and Fourier Transformed Infra Red (FTIR) spectral analysis were conducted at relatively low (0.5 µM) and high concentration of AgNO₃ (4 µM) exposed lymphocytes after 24 hours.

The heavy metals (redox active or redox inactive) may cause a rise in the production of reactive oxygen species (ROS) such as hydrogen peroxide ($\text{H}_2\text{O}_2^\circ$), superoxide radicals (O_2°) and hydroxyl radicals (HO°). Antioxidant defense system acts on ROS and result in a condition known as oxidative stress. In oxidative stress, cells display various dysfunctions caused by ROS to DNA and proteins. It is proposed that oxidative stress in cells can be partially responsible for the toxic effects of heavy metals (Ercal *et al.*, 2001). Hence the present study was aimed to quantify and determine the functional groups by HPLC and FTIR spectra of metallothioneins at low ($0.5 \mu\text{M}$) and high concentration of AgNO_3 ($4 \mu\text{M}$) exposed lymphocytes after 24 hours and compared with controls. The detailed procedure for HPLC and FTIR spectral analysis are discussed in Appendices 27 and 28 respectively.

3.4 Statistical Analysis

SPSS package version 16.0 was used for the statistical analysis of data. The statistical significant level was set at $p < 0.05$. Shapiro-Wilk test was carried out to find the normal distribution of the data ($p > 0.05$). ANOVA was performed for normally distributed data and if the data showed variation at significant level ($p < 0.05$), post - hoc test was done to know the association or difference between each group. Kruskal Wallis Test was done for non - normally distributed data and Mann-Whitney test was conducted to know the association or difference between the groups. Chi square test and Fisher's exact test were conducted for categorical variables. Spearman's rank correlation was performed to find out the association of serum metallothionein and silver levels with selected parameters.