SUMMARY AND CONCLUSION

Metallothioneins (MTs) are free radical scavengers which bind to a number of trace metals and also save cells and tissues from heavy metal toxicity. Among the most used biomarkers for pollution in the environment, MTs have been used as monitoring device or biochemical indicator of metal exposure. 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. Peripheral blood lymphocytes (PBLs) have the capacity to produce MTs as a protective response to metal exposure.

At present, the mechanisms involved in the absorption of silver into the body and accumulation in tissues are poorly defined. However, it is absorbed into the body through various organs. Silver compounds are ionized in body secretions, body fluids and moisture to produce biologically active ions (Ag^+). Ag^+ ions are the reactive species that bind strongly to metallothionein, albumins or macro globulins and are metabolized in the human body that are mostly excreted in the urine and feces. But some of the biologically active ions (Ag^+) are deposited in tissues or circulated in the biological system which would be the toxic factor causing oxidative damage to cells.

Hence it is imperative to conduct *in vivo* and *in vitro* studies in peripheral blood lymphocytes of jewellery unit workers working with silver to find out the relationship between silver and MTs which may help to understand the role of MTs in metal stress.

Objectives of the study

The study was formulated with the following objectives:

- To assess the health status of the workers in jewellery industry with special reference to silver exposure
- To characterize the metallothioneins from peripheral blood and cultured peripheral blood lymphocytes among the selected workers

- To assess the relationship between insult to exposure to hazardous levels of silver and the metallothioneins status among the workers

The study was carried out in three phases

Phase I

Assessment of the health status of workers in jewellery units

A total of 158 jewellery unit workers and 53 control group participants were selected based on specific inclusion criteria. Hematological indices, liver and kidney function parameters were assessed in them. This included assessment of complete blood count profile (total and differential count of leucocytes, red blood cell count (RBC), hemoglobin concentration (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, mean platelet volume, platelet distribution width and red blood cell distribution width in whole blood), liver function parameters namely activities of alanine amino transferase (ALT), aspartate amino transferase (AST) and alkaline phosphatase (ALP) and levels of total protein, albumin, total cholesterol and total and direct bilirubin in serum, kidney function parameters namely levels of urea, uric acid and creatinine in serum and protein and creatinine in random urine and mineral status namely calcium and phosphorus levels in serum. Protein / Creatinine (P/C) ratio of random urine samples was calculated to estimate 24 hours proteinuria. The levels of random blood glucose, metallothioneins (MT), silver, thiobarbituric acid reactive substances were assessed in serum. Correlation analyses were carried out between selected parameters in jewellery unit workers which might help to assess the interactions among them.

Salient findings

- Among the 158 workers of jewellery units 116 were married. Except 4 out of them all others had children with normal behavioral habits. This might indicate that there were no toxic effects of silver on reproduction among the jewellery unit workers. The most prominent symptom seen was abdominal pain (42%) followed by muscle weakness (32%) among the jewellery unit workers.

- Serum silver and MT levels were raised in exposed workers than controls. High levels of MT in serum suggested a cellular defense strategy against silver.

- Red blood cell indices namely red blood cell count, hemoglobin concentration and hematocrit levels were significantly decreased and Mean Corpuscular Hemoglobin (MCH) and Mean Corpuscular Hemoglobin Concentration (MCHC) levels were significantly increased in exposed workers than controls. Decrease in RBC count and increased MCH and MCHC levels might be due to exposure to silver in the jewellery units as proved by correlation analysis. Correlation of MTs with white series of cells indicated that they seem to provoke an inflammatory response against silver. But, number of neutrophils and platelets did not show any significant difference between control and exposed workers.

- Increased Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and decreased Alkaline phosphatase (ALP) activities in exposed workers than controls suggested that silver exposure alters liver enzymes metabolism. Raised level of total cholesterol in jewellery unit workers than control might indicate that silver exposure directly or indirectly alters the lipid metabolism. Serum total and direct bilirubin levels in the jewellery unit workers were found to be increased significantly compared to control. This might suggest that occupational silver exposure is associated with significant changes in hepatic clearance of bilirubin.

- Positive correlation of TBARS level with age, MT and silver might point out that age dependency and protective effect of MT induction on silver exposure might induce lipid peroxidation.
- Decreased level of calcium and elevated level of phosphorus in the study might be due to the significant effect of silver as evidenced by correlation analysis with serum silver. Increased levels of serum urea, uric acid, creatinine and urine protein / creatinine (P/C) ratio of exposed group compared to control might suggest renal dysfunction in jewellery unit workers.

Phase II

Isolation, molecular mass determination and quantification of metallothioneins from peripheral blood lymphocytes of selected workers in the jewellery units

Three workers who had high levels of serum silver and metallothioneins were selected. Ten ml of blood samples were collected from them to isolate peripheral blood lymphocytes (PBL). Cell lysate was loaded on Sephadex G-75 column and eluted fractions containing proteins were estimated in UV spectrophotometer at 250 and 280 nm. The ratio of absorbance at 250 / 280 nm more than 1 indicated the presence of mercaptide bonds. The corresponding eluted fractions were pooled together and loaded on SDS-PAGE to know the molecular weight of the MT, quantified by High Performance Liquid Chromatography. Functional groups in MT protein were identified by Fourier Transformed Infra Red spectroscopic methods. Total sulfhydral content of the concentrated MT was estimated.

Salient findings

- The molecular weight of proteins obtained by SDS PAGE were 21.5 kDa and 42 kDa in exposed group and 47 kDa for control group. These fractions might indicate the formation of multimeric complex of multiple sulfhydryl groups. Sulfhydryl content of isolated metallothioneins was also higher in exposed group than control. Percent of MT in the samples was estimated by HPLC as 40.3% and 11.27% in exposed and control groups respectively which might confirm that MTs might be induced in response to metal exposure.

Phase III

Expression of metallothioneins in the cultured lymphocytes of the selected workers on exposure to silver ions

PBLs were isolated from ten ml of blood samples from voluntary donors of control group (n = 3) for cell culture study. Isolated PBLs were cultured in RPMI 1640 medium with 0.5, 1, 2 and 4 µM concentrations of $AgNO_3$. Cytotoxicity was assessed in cultured lymphocytes treated with different concentrations of $AgNO_3$ (0.5, 1, 2 and 4 µM) after 3, 6, 12, 24 and 48 hours time intervals by MTT assay. Cell viability was assessed after 24 hours using 0.4% trypan blue. Uptake of 4 µM $AgNO_3$ was observed

after 24 hours in cultured lymphocytes using Transmission Electron Microscope. MT was isolated from cultured PBLs after 24 hours by gel filtration chromatography, quantified by HPLC and functional group was identified by FTIR. Total sulfhydryl content of MT in cultured PBL was estimated. Free radical scavenging activity of concentrated MT from different concentrations of $AgNO_3$ treated cells was estimated.

Salient findings

- Peripheral blood lymphocytes (PBL) treated with higher concentrations of $AgNO_3$ (2 and 4 µM) showed noticeable decrease in viable cells (80% and 67% respectively) after 24 hours than control that was evidence by PBL staining with trypan blue. The extent of cell death might be due to the oxidative stress. The extent of cell survival on exposure to different concentrations of $AgNO_3$ (0.5, 1, 2 and 4µM) by MTT assay showed that silver nitrate caused a dose and time dependent loss of cell viability in PBLs with ~50% as the highest dose (4 µM) after 48 hours.

- Transmission Electron Microscopic images of 4µM $AgNO_3$ treated PBLs showed the presence of electron-dense nanosilver precipitates after 24 hours. This precipitate was absent in control cells.

- HPLC spectral analysis of MT in the samples recorded 45.86 % and 47.11 % present in 0.5 µM and 4 µM $AgNO_3$ treated cells respectively. Cultured control PBLs did not show MT peak in the HPLC profile. This result provided evidence that MTs could be induced by metal exposure.
- MT scavenged the free radicals - almost 54% of super oxide radicals followed by 45% of DPPH and 38% of ABTS. MT exhibited least scavenging activity on H_2O_2 (23%).

Conclusions drawn from the findings of the present investigation

High levels of metallothioneins and silver in serum might indicate that MT provided cellular defense strategy against silver. The noticeable changes in hematological indices, liver and kidney function parameters due to the exposure of jewellery unit workers to silver might have negative impact on human health. Correlation

of silver and metallothioneins with selected liver and kidney function parameters suggested that uptake of Ag-MT complexes by kidney cells derived from the liver and an increase in active Ag accumulated in the kidney by the excessive degradation of Ag-MT might be the responsible factors for reduction in kidney function.

Elevated levels of metallothioneins in $AgNO_3$ treated cells might indicate their involvement in the scavenging and detoxification of silver. TEM analysis of PBLs showed the accumulation of electron dense precipitates after 24 hours of incubation in the presence of 4μM $AgNO_3$. Free radical scavenging potential of isolated MT from $AgNO_3$ treated PBLs showed maximum scavenging activity on superoxide radical and least scavenging activity on hydrogen peroxide radicals.

The study clearly suggested that expression of MT in PBLs might serve as a biomarker of silver exposure.

Scope for future studies

- ✓ A longitudinal study could be attempted in animal model on silver toxicity and metallothioneins status.
- ✓ A study on characterization of different isoforms of metallothionein from jewellery industry workers could be conducted to find the forms of MT expressed more in metal exposure.
- ✓ A study could be conducted on cultured $AgNO_3$ treated PBLs to monitor the role of MT in cell proliferation and cell differentiation.
- ✓ A relative order of affinity of metallothioneins with different metals and binding ability of different isoforms of MT with metals could be studied in cultured cells.