Free Radical Scavenging Activity of Metallothioneins from Peripheral Blood Lymphocytes of Gold Jewellery Karigars

Shalini K.V. and Jeyanthi G.P.
Department of Biochemistry Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher education for Women, Coimbatore-641 043, Tamilnadu, INDIA

Abstract

Metallothioneins (MT) are cysteine rich, low molecular weight metal binding proteins involved in detoxification and homeostasis of metals. Due to a variety of chemical stresses MTs are stimulated and can be used to assess human exposure to occupational agents. Human Peripheral Blood Lymphocytes (PBLs) have the ability to produce MTs against occupational exposure to silver (Ag). In the present study, PBLs were isolated from the blood of gold jewellery unit workers and MT was separated by sephadex G-75 gel filtration. Molecular mass of MT was determined by SDS-PAGE using silver staining method from the collected fractions. HPLC was performed to measure the levels of MT from the collected fraction. It is suggested that the elevated levels of MT in PBLs can be used as specific indices of silver exposure.

Keywords: Metallothioneins, peripheral blood lymphocytes, silver.

Introduction

Heavy metals are described as common transition metals that have the potential to cause harm to the environment and most of the heavy metal exposure to humans occurs through occupational settings. Jewellery making one of the world's oldest manufacturing sectors involves some hazardous processes. Alloys are added during the manufacturing process of gold which help to give hardness, to make the gold jewellery stronger and would change the colour. Most often, pure gold is alloyed with copper, zinc and silver in varying proportions to produce the wide range karat gold. Goldsmiths are also known to use potent toxic chemicals like amile nitrates (polishing compounds), ammonium chloride, aniline dyes, cadmium, cadmium bicarbonate, copper sulphate, mercury, potassium cyanide, potassium hydroxide, potassium nitrate, silver, silver nitrate and nitric acid. These chemicals are used for melting, refining, welding and polishing the jewellery.

During the manufacturing processes, formation of fumes and dusts which consist of metals and hazardous compounds is common. 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.

Silver (Ag) is a xenobiotic element with no recognized trace metal value in the human body which is used in higher concentration for gold fabrication. At present, the mechanisms involved in the absorption of Ag 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 and moisture to produce biologically active ions (Ag^+). Ag^+ ions are the reactive species bind strongly to metallothionein, albumins and macro globulins are metabolized in the human body which is mostly excreted in the urine and feces. But some of the biologically active ions are deposited in tissues or circulated in the biological system which would be the consequence toxic factor causing oxidative damage to cells[1,2,3].

Metallothioneins (MTs) are free radical scavengers which bind to a number of trace metals and also save cells and tissues from heavy metal toxicity. They are highly conserved family of low molecular weight cysteine rich and closely related stress response proteins with molecular weight from 5- 16 kDa. A wide range of molecular weight proteins obtained due to the increasing concentration of the heavy metals[4]. MT consisted of more than 30% of cysteine residues directs their metal binding properties[5]. MTs are synthesized due to a variety of cellular stressors and have been found in intracellular as well as extracellular spaces. It has been found that there are 250 various structural forms of MTs[6].

The functional importance of MT is still under question. The function of mammalian MTs are hypothesized by various researchers revealed that it was involved in homeostasis and detoxification of metals also it protect the cells and tissues from the oxidative stress, maintain the intracellular redox balance and regulate the cell proliferation[7,8]. Several reviews exposed about this exclusive molecules have expanded the inquisitiveness of researchers to study the secrecy of MTs for over five decades[9,10,11,12,13,14]. The metal ions have different relative order

of attraction towards MTs such as Hg > Ag >> Cu > Cd > Zn reported in several *in vitro* studies [15].

Metals bound to MT are considered less labile and thus less toxic, but the exact mechanism of protection against metals is complex. Indeed, the mechanism of action of MT depends on the degree of heavy metal exposure of tissues and the MT response[16].

There are several studies have been carried out in peripheral blood lymphocytes (PBLs) to know the expression of MTs *in vivo* and it was found that MTs can be induced appreciably with metal exposure[17,18,19]. Hence, the present study was aimed to isolate, determine the molecular mass and quantify the MT from PBLs using gel filtration, SDS PAGE and HPLC methods respectively.

Material and Methods

Subjects and Area: The present study is part of a research that has been performed in Coimbatore (Kuniyamuthur, Saibaba Colony and R.S Puram) Tamilnadu, during the year 2012. The study consisted of 45 jewellery unit subjects aged 25–45 years who had resided and worked in the same place for many years and were asked to participate in the study. These 45 jewellery unit workers were considered as "exposed group". Forty three participants of the same age who lived in the same area with no relation to making of jewellery were considered as "control group." An informed written consent was obtained from each subject of control and exposed groups and blood collection was performed only after permission of the ethical committee of Avinashilingam Institute for Home science and Higher Education for Women, Coimbatore, Tamilnadu (HEC.2011.27). The study consisted of selected inclusion and exclusion criteria. Workers with minimum one year of exposure were included and those with known history of diabetes mellitus, liver diseases, blood transfusion and any other pathological conditions were excluded from the study. Each participant of the study received a questionnaire and orally was explained in local language for illiterates, to get their personal details.

Sample collection and analysis: Fresh blood samples were preserved in ice bags during the collection of samples. Vacutainers (AcCuvet-PLUS) with clot activator specific for serum were used to assess the metallothionein content by modified method of Viarengo et al (1997). Serum silver concentration was determined by Flame Atomic Absorption Spectrophotometric technique (SHIMADZU, AA- 7000), Height and weight were measured by standard method. Systolic and diastolic blood pressure was measured using Omron Blood Pressure Monitor: HEM 7112. Blood pressure was expressed in mm Hg.

Estimation of metallothionein: Serum vacutainers were centrifuged at 3000 rpm for 10 minutes and the collected serum was stored in cryovials at -20^{0}C. To analyze the metallothionein, high molecular weight proteins of serum was precipitated by using 200µl of ice cold absolute ethanol and 16µl of chloroform per 200µl serum. Further analysis was done by Viarengo *et al* method[20]. GSH was used as a standard for quantification of MT in the sample because GSH contains one cysteine per molecule. To determine the levels of MT in the samples can be calculated by the formula:

$[(Abs^{MT412}/εGSH)/20] \times 6800 \times 4.5$ in which (Abs^{MT412} = OD values of metallothionein samples, εGSH = Extinction coefficient for GSH (Constant), 20 = Number of cysteine residues in 1 molecule of metallothionein, 6800 = Molecular weight of metallothionein, 4.5(ml) = Final volume of DTNB reaction).

Protein isolation and molecular mass determination: The donors who had high levels of metallothionein and silver content in serum were selected. The peripheral blood lymphocytes 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 1077density-gradient centrifugation from freshly collected venous blood. Isolated lymphocytes were centrifuged at 2000 rpm for 10 minutes to obtain the pellets. Cell pellets were lysed in cooled lysing buffer contains NET (100mM NaCl, 1 mM EDTA, 20 mM Tris) of 0.5% TritonX 100 (v/v) and protease inhibitor (Thermoscientific). Cells were kept in lysing buffer for 15 minutes and cell debris was pelleted by centrifugation (15,000g, 15min, 4ºC). The supernatant was applied to a 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) and eluted with same buffer at a rate of 15 ml/h. The fraction volume was 1.5 ml. 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 was further confirmed by HPLC analysis. Absorbance (OD value) was less at 280 nm because they are devoid of aromatic amino acids and at 250 nm the absorbance was high as the presence of mercaptide bonds with metals[21].

Those fractions with the ratio of absorbance at 250 nm/280 nm more than 1 were pooled together and run in sodium dodecayl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to determine the molecular mass of protein. Proteins were electrophoresed using the vertical stab gel apparatus with 12 % polyacrylamide gels containing 2% SDS, acrylamide (95 %) and NN'- methylenebisacrylamide (5 %) specially purified for electrophoresis (Sigma Chemicals). DTT (Di-Thrio-Threotal) were used for reduction of protein disulfide bonds in SDS PAGE. Bromophenol Blue was used as a marker for migration distance. The pooled samples were heated in a water bath (95ºC) for 3 minutes and loaded on SDS PAGE gel according to the modified method of Aoki and Suzuki[22]. After electrophoresis, proteins were stained with silver stain by modified method of Merril *et al*[23].

Protein analysis: A rapid and sensitive High Performance Liquid Chromatography (HPLC – Shimadzu LC 10 AVP) method for the determination and quantification of metallothionein (MT) in peripheral blood lymphocytes (PBL) has been studied. Methallothioneins (MTs) were separated in reverse phase C-18- phenomenex LC column, eluted through a linear gradient of 2% THF (T*etrahydrofuran*) in Water **:** 1 % THF in Acetonitrile (40:60) at the flow of 2.0 ml/min with a column temperature of 25º C. Furthermore, the content of MT was calculated by protein peak area in a short time (about 45 min). 10 µl of the filtered sample was injected to the automatic injector using a Micro syringe (1-20µl, Shimadzu). The class VP integration software was used for the data analysis. Separation and quantification of metallothioneins were carried out in minimum of three independent experiments of control and exposed groups.

Statistical analysis: SPSS package version 16.0 was used for the statistical analysis of data and draws the graph. The statistical significance level was set at $p<0.05$. Shapiro-Will test was carried out to identify the normal distribution of the data ($p>0.05$). Independent sample T test was performed for normally distributed data or Mann Whitney U test was conducted for non - normally distributed data to know the statistical significance of the data. Spearman's rank correlation was performed to find out the association between serum metallothionein and serum silver levels. A linear regression analysis was used to verify the influence of Ag on serum MT concentration. The serum silver concentration was treated as dependent variables, while serum metallothionein concentration as independent values.

Results and Discussion

The socio-demographic characteristics of exposed and control group are listed in table-1 which confirmed that there were no significant differences in height, weight, age distribution, systolic and diastolic pressure between the exposed workers and control group. Jewellery industry workers in this study had been exposed to hazardous compounds during the manufacturing process in which silver is used in higher concentration (5%). Silver is considered as a xenobiotic compound for human body. Therefore the study was more concentrated on silver exposure of jewellery industry workers who had been exposed to this compound daily over extended period of time. Figure- 1 indicates that serum silver level was significantly raised ($p<0.05$) in exposed group than in control group. Similar result was reported on the oral toxicity of silver nanoparticle (56 nm) over a period of 90 days in f344 rats, which showed a dose dependent accumulation of silver in all tissues[24]. In the present study serum MT level was significantly increased ($p<0.05$) in exposed group compared to control group- figure-2. A similar outcome was observed in the gill of two varieties of mollusks[25].

A significant positive correlation ($r = 0.565$, $p<0.05$) between the levels of serum silver and serum metallothionein was observed in figure- 3. The linear regression analysis revealed that serum silver level was statistically associated with the serum metallothionein shown in table-2. These results were supported by a study on MT gene expression in PBLs from cadmium exposed workers described that both basal and induced MT expression levels were significantly correlated with blood and urine cadmium levels[26].

Table-1
Socio-Demographic Characteristics of Jewellery Industry Workers

Groups	Height (cm) ◊	Weight (Kg) *	Age ◊	Blood pressure (mmHg)	
				Systolic pressure ◊	Diastolic pressure *
Control (n=43)	156 (160-154)	68.42 ±9.70	35 (44-27)	119 (120-113)	75.05 ±7.75
Jewellery industry workers (Exposed) (n = 45)	157 (161-153)	67 ±12.3	36 (41-33)	120 (122-117)	78.82 ±10.27
p Value(< 0.05)	0.160 ⇑	0.674 ˢ	0.253 ⇑	0.223 ⇑	0.350 ˢ

◊= median value (Inter quartile range); *= mean value ±standard deviation; ⇑= Mann Whitney U test computed for groups with unequal sized samples; ˢ = Independent sample t test computed for groups with unequal sized samples.

Table-2
Linear Regression Analysis of Serum Metallothionein in Occupational Silver Exposed Workers

Ag (µg/l)	Variable	Unstandardized Coefficient		Standardized Coefficient	t	Sig
		B	Std.Error	Beta		
	(Constant)	-0.558	1.854		-0.301	0.764
	MT(nmol/)	1.948	0.324	0.560	6.005	0.000

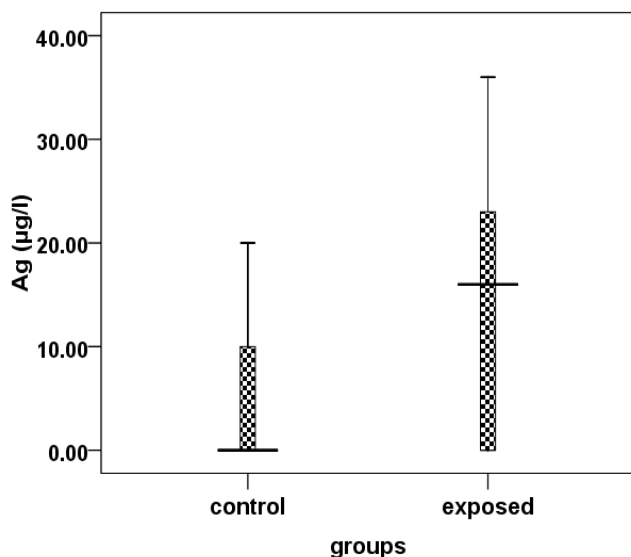The B is unstandardized coefficient and β is the standardized coefficient.

Figure-1
Levels of Silver among Workers in Jewellery Industry

Exposure scenario of the examined population in which serum silver levels were studied in exposed (n=45) and Control group (n= 43) participants, indicated the serum silver level was significantly raised (p<0.05) in exposed group than control group
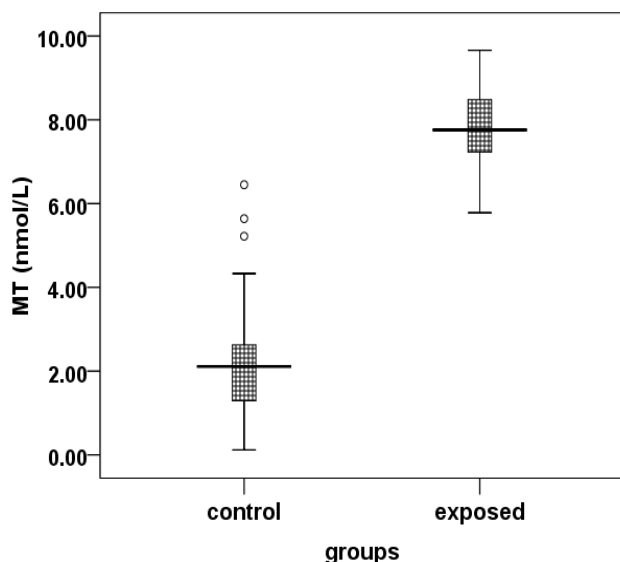

Figure-2
Activity of Metallothionein among Workers in Jewellery Industry

Box plot of the levels of metallothionein in serum of exposed (n=45) and Control group (n= 43) participants, showed the MT level was significantly increased (p< 0.05) in exposed group compared to control group.
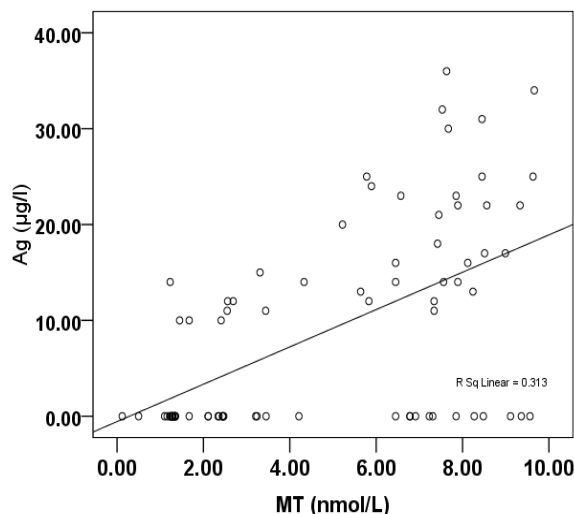

Figure-3
Correlation between Serum Silver and Metallothionein Levels in Occupational Silver Exposed Workers

The serum silver and metallothionein levels were measured as described in the text. The correlation between silver and metallothionein levels was calculated using linear regression analysis.
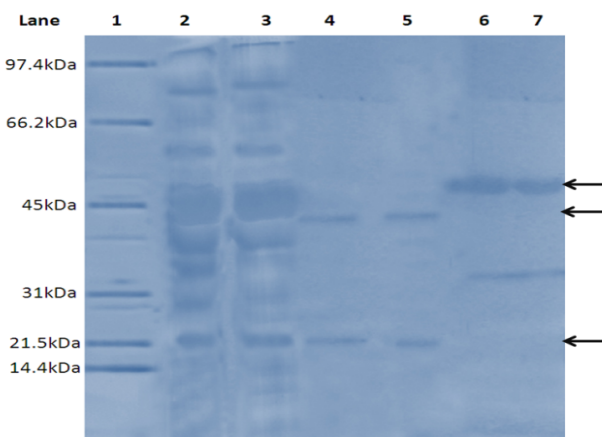

Figure-4
SDS-PAGE Protein Profile of Peripheral Blood Lymphocytes of Silver Exposed Workers

Lane: 1 shows protein standard marker
Lane: 2 and 3 shows the protein profile of PBL of control and exposed group respectively
Lane: 4 and 5 shows the gel filtered protein profile of PBL of exposed group
Lane: 6 and 7 shows the gel filtered protein profile of PBL of control group.
The arrow mark indicates the protein bands of 21.5, 42 kDa of exposed workers and 47 kDa of controls
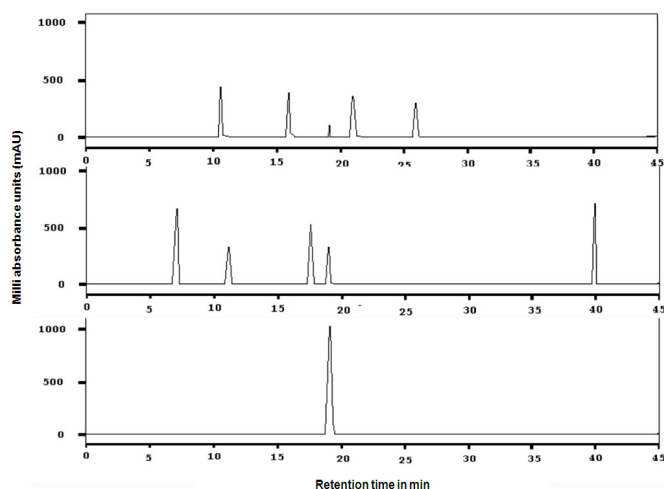
Figure-5
HPLC Spectrum of Metallothionein in PBLs of Exposed Workers and Controls

The retention time for metallothionein of standard (sigma), exposed and control samples were 19.02, 19.12, and 18.97 min, respectively and the detection wavelength was 254 nm.

Peripheral blood lymphocytes were collected from the selected exposed and control group participants. Then the cell extract was eluted through gel filtration chromatography (Sephadex G-75) and the absorbance was measured at λ = 250 nm and λ = 280 nm. As expected, the low molecular-weight protein showed a high absorbance at 250 nm, a feature indicating the binding of silver to MT and low absorbance at 280nm confirmed devoid of aromatic amino acids as it had already been shown for MT from various species[27]. The fractions with 250/280 absorbance ratio more than 1 were collected and assayed for sulfhydryl groups with the Ellmann method in collected fractions[28]. The results confirmed the presence of numerous sulfhydryl groups in the samples.

The standard protein markers (14.4- 97.5 KDa) purchased from the Biorad was used as reference standards in order to identify molecular mass of protein from collected fraction of PBLs of exposed and control group. The SDS-PAGE profile is shown in figure- 4 and the molecular mass determined by SDS PAGE was about 21.5kDa and 42 kDa for the exposed group and 47kDa for the control group.

The high molecular weight protein obtained might be due to the formation of multimeric complex of multiple sulfhydryl groups. A study on the identification of MT in *Pleurodeles walti* showed that the protein displayed its apparent molecular weight of 28 kDa. This result does not fit well with the expected 6 Kda MT molecular weight. This discrepancy can be explained taking into account the unusual properties of MT. The multiple sulfhydryl groups of MT in fact lead to the formation of multimeric complexes and then to form as high molecular weight protein[29].

In figure-5 HPLC spectrums showed the retention time for horse kidney metallothionein standard (Sigma), exposed and control were 19.02, 19.12, and 18.97 min, respectively. The detection wavelength was 254 nm and percentage of MT in the sample could be calculated by area under the curve method [(A= X x Y x P/ Z x S) (X = Concentration of the standard marker injected, Z = Area given by the standard compound, Y = Area given by the marker compound in the sample profile, S = Sample concentration, P = Purity of the marker compound taken ,A = % of marker compound in the sample)]. This was estimated as 40.3 % and 11.27 % present in exposed and control samples respectively. This result revealed that elevation of metallothionein in exposed group compared to control group. A novel report of MTs in fish bile was studied, where three methods (1D/2D gel electrophoresis, spectrophotometric analyses and SEC-HPLC-ICPMS) were carried out to verify the presence of MT in which all three methods showed the presence of MT[30].

Conclusion

The significant level of serum silver seems to induce expression of MT in PBLs. The present study showed that raised level of MT as analysed by spectrophotometric and HPLC techniques of exposed group than control group in PBLs might serve as a biomarker of silver exposure. Elevated levels of MT might indicate their involvement in the scavenging and detoxification role of silver.

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