

RESULTS AND DISCUSSION

Silver (Ag) is a non - vital metal used in a wide variety of applications. Since the time of its wide spread uses, the systems participation in the uptake of silver in the human cells was poorly known (Bertinato *et al.*, 2010).

Previous studies have shown that silver is considerably less toxic than any other heavy metals. However high levels of silver exposure has been associated with impaired immune function (Gamelli *et al.*, 1993) and also depleted glutathione levels (Fechter *et al.*, 2008). In addition, animal and cell culture studies showed that silver exposure may lead to reduced cell proliferation, increased lipid peroxidation and undesirable effects on the central nervous system (Powers *et al.*, 2010).

Silver exposure occurs to human through occupational settings or from consumption of Ag-containing medicines which can result in deposition of significant amounts of Ag in the eyes, skin and other tissues. Moreover, this metal occurs as a contaminant in air and food and enters the body by inhalation, dermal absorption and ingestion (Drake and Hazelwood, 2005).

Jewellery making is one of the oldest manufacturing sectors in which alloys are added during gold fabrication. Various alloys are used during the manufacturing process of gold such as copper, zinc and silver in which silver is used in higher concentration and it is also a xenobiotic compound to the human body. Hence it is imperative to estimate the level of silver in biological samples to know the health status of jewellery unit workers.

Metallothioneins (MTs) are free radical scavengers which bind to a number of trace metals and also save cells and tissues from heavy metal toxicity. Peripheral blood lymphocytes have the capacity to produce MTs as a protective response to metal exposure. Heavy metals are one of the factors for induction of MTs in lymphocytes which resembles that in liver (Yeh and Yen, 2005). Hence *in vivo* and *in vitro* studies were conducted in peripheral blood lymphocytes of jewellery unit workers to find out the

relationship between silver and MTs which might help to understand the role of MTs in metal stress.

The results of the present study are discussed under the following headings:

PHASE I

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PHASE I

4.1 Health Status of Workers in Jewellery Units

Silver is the ideal industrial material. It has been used in wide variety of applications such as pharmaceuticals, electrical, chemical catalyst, industrial, printed circuitry, superconductors, electroplating, brazing, soldering, coins, photography, silverware and jewellery (Panyala *et al.*, 2008). A wide variety of uses of silver in industries allows its exposure through various routes of entry into the body. Soluble and insoluble silver compounds have the potential to cause adverse effect to the human health (Weir, 1979; Howe and Dobson 2002).

Silver can gain entry into the body through inhalation, ingestion and dermal absorption of occupational settings. Acute symptoms of over exposure of silver nitrate causes diarrhea, decreased blood pressure, stomach pain and reduced respiration. A chronic symptom of low level exposure to silver salts causes alteration in blood cells and fatty degradation of liver and kidneys (Venugopal and Luckey, 1978). Long term inhalation and ingestion of soluble silver compounds may cause argyria and argyrosis (Nordberg and Gerhardsson, 1988; Fung and Bowen, 1996; Gulbranson *et al.*, 2000).

One fifty eight male workers (20-50 years) working in jewellery units designated as exposed groups and 53 unexposed men (20-50 years) as controls were selected for the study. One fifty eight male workers were further categorized into two groups as occasional (n= 85) and frequent workers (n= 73). The jewellery workers live and work in small rooms with their family and most of them do not wear protective mask, shoes, personal protective clothes and gloves. They seemed to follow unhygienic practice which increases the risk of human health.

4.1.1 Demographic profile of the selected workers employed in jewellery units

Table 4A and 4B illustrate the profile of selected jewellery unit workers as per the information obtained by administering the questionnaire.

Table 4A
Demographic Profile of the Selected Workers Employed in Jewellery Units

Factors	Control group (n= 53)	Jewellery unit workers (n= 158)
Educational qualification		
a) < 5 th Standard	9	36
b) 5 th – 12 th Standard	33	109
c) Graduates	11	13
Marital status		
a) Married	37	116
b) Unmarried	16	42
Work experience		
a) 1-10 years	18	47
b) 11-20 years	23	71
c) >20 years	12	40
Economic background		
(Income/Month in Rupees)		
a) Up to 5000	18	67
b) 5001 to 8000	24	66

c) 8001 and above	11	25
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Life style

a) Consumption of fresh	16/37	65/93
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vegetables and fruits (Yes/No)

b) Exercise (Yes/No)	5/48	27/131
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c) Limited intake of fats (Yes/No)	21/32	49/109
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Educational qualification

Among the jewellery unit workers only 13 were graduates which might point out ignorance of participants about the pollution and toxicity caused by this industry.

Marital status

Hundred and sixteen were married among the 158 workers of jewellery units. Except 4 out of 116 jewellery unit workers, all others had children with normal behavioral habits. This indicated that there were no toxic effects of silver on reproduction among the jewellery unit workers.

Unlike other metals, metallic silver is not toxic to humans and is not known to cause cancer, reproductive or neurological damage and other chronic adverse effects. Prolonged exposure to silver dust or fumes can cause mild effects on human health. But Ag^{2+} (ionized form of silver) is found to inhibit human sperm creatine kinase activity (required for high energy) in a dose dependent manner. Even though it was identified as heavy metal and was correlated with an overall decrease in male fertility, the exact mechanism is not clearly known (Ghaffari and Motlagh, 2011).

Work experience

Based on the duration of the work experience, 158 jewellery unit workers were categorized in to 1-10 years (n = 47), 11-20 years (n = 71) and more than 20 years (n = 40) of exposure in this unit.

Economic background

Among the exposed group participants, 67 had monthly income of up to Rs. 5000, 66 had Rs. 5001- 8000 and 25 had an income above Rs. 8001.

Life style

Sixty five members of jewellery unit workers had reported to have fresh fruits and vegetables every day, 27 reported to do exercise every day and 49 reported to limit to have fat foods among the 158 participants for the study.

Table 4B presents the details of age and alcohol intake among the jewellery unit workers

Table 4B (Contd....)

Demographic Profile of the Selected Workers Employed in Jewellery Units

Groups	Age*	Frequency of alcohol consumption /Week*
Control group (n = 53)	35 (44 - 27)	3.0 (21.50 – 0.00)
Exposed group (n =158)	36 (42 - 32)	5.0 (25.00 – 0.00)
p value (>0.05)	0.200	0.344

*= median value (inter quartile range)

Mann Whitney test computed for groups with unequal sized samples

Age

The distribution of age among the control and jewellery unit workers is summarized in table 1B. Median and inter quartile range are shown in table 1B. The present study indicated that there was no significant difference ($p > 0.05$) in the distribution of age between the control and exposed groups.

Alcohol consumption

Alcohol consumption and smoking habits are considered as the confounding and environmental factors which might interfere in the findings of the present study. Hence smoking status (current smokers, passive smokers and ex smokers) and alcohol consumption were documented for all the subjects.

The impact of alcohol consumption was found in major diseases such as cancer in nasopharynx, oropharynx, colon, rectum, breast, liver and oesophagus, hypertension and ischaemic heart disease, diabetes mellitus, unipolar depressive disorders, haemorrhagic stroke, epilepsy, cirrhosis of the liver and lower respiratory infections (Rehm *et al.*, 2010). The present study showed that there was no significant difference ($p > 0.05$) among alcoholic intake between control and exposed groups which indicated that alcohol consumption did not confound the study results.

4.1.2 Smoking habits of workers among jewellery units

Pack years (smoking habit)

Smoking is considered to be a vital factor for many pathological conditions. It influences both innate and adoptive immune responses. It may lead to high levels of numerous pro-inflammatory cytokines, anti-inflammatory cytokines and elevated levels of IgE causing atopic diseases and asthma (Arnson *et al.*, 2010).

Silver acetate chewing gum is used as a smoking deterrent, which may lead to accumulation of silver in skin and cause the development of argyria (Jensen *et al.*, 1988). The present study did not indicate any significant difference ($p > 0.05$) in current smoking habit between the control and jewellery unit workers. Current smoking habit of the selected participants was expressed in pack years (Section 3.1.4).

Ex smokers

Ex smokers include people who have previously been smokers. The present study consisted of 14 members who were former smokers in jewellery units. There was no significant difference ($p > 0.05$) observed in the ex smoking habit of control and exposed group workers of the study.

Passive smokers

Passive smoking is the inhalation of smoke (second-hand smoke), or environmental tobacco smoke by persons other than active smoker. Passive smoking may cause heart diseases (Wells, 1994), obstructive respiratory disease (Dayal *et al.*, 1994), fertility problems and even breast cancer in women (Repace, 1981). There were 31 out of 158 participants as passive smokers among the exposed group of the study. In this study, there was no significant difference ($p > 0.05$) in the passive smoking habit of control and exposed group.

The present study showed that there was no significant difference ($p > 0.05$) among smoking habit of current smokers, passive smokers and ex smokers between control and exposed groups which indicated that smoking habit did not confound the study results.

Chewing tobacco users

Eighteen members of exposed group were found to be chewable tobacco users in the present study. Chewing tobacco seemed to cause minor problems such as tooth stains, gum problems, losing taste for food, bad breath and many health problems. The most serious problems are white patches and sores inside the mouth which leads to cancer (Kademani, 2007). The present study showed that there was no significant difference ($p>0.05$) in the chewing tobacco habit between the control and exposed group workers.

Table 5 presents the details of ex smokers, passive smokers and chewing tobacco users of jewellery unit workers.

Table 5
Smoking Habit of the Jewellery Units Workers

Factors	Control (n=53)	Jewellery unit workers (n=158)	p value (p < 0.05)
Pack Years*	0.74 (2.5 - 0.00)	0.76 (2.3 - 0.37)	0.549
Ex smokers			
Yes	7 (13.2%)	14 (8.86 %)	0.360 \$
No	46 (86.8%)	144 (91.14%)	
Passive smokers			
Yes	11 (20.8%)	31 (19.6%)	0.858 \$
No	42 (79.2%)	127 (80.4%)	
Chewing tobacco users			
Yes	2 (3.77%)	18 (11.39%)	0.107 \$
No	51(96.23%)	140(88.61%)	

* Values are expressed as median (Inter quartile range)

Mann Whitney test computed for groups with unequal sized samples

\$ F Values for Chi square test

Besides drug or industrial exposures, silver may be ingested with foods (wheat flour, bran and mushroom), milk and water. Few studies indicated that silver products have the potential risk for the developing fetus in pregnant women (Gulbranson *et al.*, 2000; Landsdown, 2010). High concentration of silver exposure was found to cause

glomerular damage and proteinuria. Metal fume fever and chemical pneumonitis seemed to arise due to inhaled silver (Sullivan and Krieger, 2001). Topical use of silver nitrate in burns was found to lead to methemoglobinemia (Sharma *et al.*, 2006).

Since none of the workers in the present study consumed any colloidal silver nor silver containing medicines for illness, silver exposure in them might be believed to be mostly due to silver from the jewellery units.

4.1.3 Professional background of workers employed in jewellery units

Table 6 shows the professional background of the jewellery unit workers

Table 6		
Professional Background of Workers Employed in Jewellery Units		
Jewellery unit workers (n=158)		
Factors	Occasional workers (n=83)	Frequent workers (n=75)
Working Hours (Days / Month)		
a) ≤ 15 days	72 (86.75%)	-
b) > 15 days	11(13.25%)	75 (100%)
KDM Gold makers	62 (74.7%)	45 (60%)
Knowledge about emission (Yes/No)	41/42	26/49
Precautionary measures (Yes/No)	32/51	23/52

Working days / month

Among the 83 participants of occasional workers, 86.75% and 13.25% of the members worked for less than 15 days and more than 15 days respectively per month. Hundred percent of frequent workers worked more for than 15 days per month. This might indicate that frequent workers group was exposed to pollutants more than the occasional workers group. Working hours/day of exposed groups could not be calculated due to lack of information from them.

KDM gold makers

KDM gold jewellery was soldered with cadmium. Currently cadmium is banned in many countries because it creates toxic fumes when it is melted and is hazardous to human health. Currently, 916 gold is called as KDM gold which consists of 91.6% pure gold and the rest includes metals namely zinc, copper and silver. Among the 158 jewellery unit workers, 107 participants were making KDM gold jewellery as elicited in the questionnaire of the study.

Knowledge about the emission

Sixty seven (42.4%) workers out of 158 participants of jewellery units were aware of the emission of pollutants in this occupation among whom 41 (49.4%) and 26 (34.67%) members were from occasional and frequent workers group respectively.

Use of precautionary measures

Among the exposed group workers (n =158), 55 (34.8 %) participants have used protective measures during working hours.

4.1.4 Protective measures in use among jewellery unit workers

Table 7 shows the details of the protective measures regularly used by the jewellery unit workers (n = 158).

Table 7

Protective Measures in Use among Jewellery Unit Workers (n=158)

Protective measures	Yes (%)	No (%)
Respiratory mask	-	158 (100%)
Gloves	12 (7.6%)	146 (92.4%)

Safety glasses	7 (4.4%)	151 (95.6%)
Chappals	23 (14.6%)	135 (85.4%)
Avoidance of eating during work hours	8 (5.1%)	150 (94.9%)
Avoidance of drinking during working hours	5 (3.2%)	153 (96.8%)
All the above protective measures	-	158(100%)

Prevention of occupational hazards is much more effective and cheaper rather than as control solutions of already existing hazardous situations. The personal protective measures such as air filters, respiratory protective equipments, shoes and gloves reduce the effects of hazardous pollutants to human at work place. Ingestion of hazardous compounds that occurs through drinking and eating in the work place should be forbidden (World Health Organization, 1999). According to Nij *et al.* (2003), the combined use of more than one protective measure can reduce exposures to acceptable levels of pollutants.

4.1.5 Symptoms reported by jewellery unit workers

Table 8 features the symptoms reported by selected jewellery unit workers which were elicited by administering questionnaire.

The most prominent symptom seen in exposed group workers was abdominal pain (42%) followed by muscle weakness (32%) among the jewellery unit workers in the present study. Out of 158 jewellery unit workers, 18% people reported eye irritation and throat pain and 2% persons reported wheezing and allergy. Twenty three per cent and 16% people informed head ache and dry skin respectively.

Oral consumption of colloidal silver may result in agranulocytosis, hemolysis, pleural edema and coma. Ingestion of large dose of inorganic silver may lead to burning of throat, abdominal pain, diarrhea, vomiting and convulsion as reported by Wadhera and Fung (2005).

None of the jewellery unit workers showed argyria (discoloration of the skin) and argyrosis (discoloration of the eyes) which is a symptom of severe silver toxicity. Forty eight (30%) out of 158 workers were absolutely normal as Sanchez-Huerta *et al.* (2003) opined that there is a wide variation in individual susceptibility to silver intoxication.

Some individuals have clinical symptoms due to silver exposure at an early stage and may begin at a low dose whereas, in others, symptoms may not appear until high dose. A number of factors influence on metals to produce toxic effect on human body such as solubility, binding ability with protein, metabolism and excretion.

Table 8
Symptoms Reported by Jewellery Unit Workers

Symptoms/History	Percentage	
	Control group (n = 53)	Exposed groups (n = 158)
Throat pain	9	18
Nausea	-	5
Abdominal pain	-	42
Headache	11	23
Dry skin	6	16
Dizziness	-	4
Eye irritation	-	18
Allergies	2	2
Muscle weakness	-	32
Wheezing	-	2
Jaundices	-	3
Kidney stones	-	6
Redness of skin	-	4
Decreased appetite	4	17
Absolutely normal	64	30

Continuous exposure to silver compounds for prolonged period of time may cause blue-gray pigmentation on skin and body tissues. Exposure to fumes and dust of silver nitrate and silver oxide at high dose may cause lung and throat irritation, breathing problems, vomiting and stomach pain. Skin contact with silver compounds may cause mild allergic reactions such as rashes, redness of skin and swelling as reported by Agency for Toxic Substances and Disease Registry (1990).

According to Trickler *et al.* (2010), silver nanoparticles may interact with cerebral microvasculature and produce a proinflammatory cascade which may further induce brain inflammation and neurotoxicity. Gliga *et al.* (2014) reported that silver nanoparticles (10 nm) were cytotoxic to human lung cells and the toxicity observed was associated with the level of intracellular silver (Ag) released in the cells.

There was no past history of chronic or acute ill health among workers in jewellery units. However five workers had jaundice and nine had kidney stones. Hence they were eliminated from the study (Section 3.1.2, Figure 6).

4.1.6 Anthropometric measurements of jewellery unit workers

Jewellery unit workers and the control group men did not show any significant differences in height and weight ($p > 0.05$) of the study. Sung *et al.* (2009) reported that the subchronic inhalation toxicity of silver nanoparticles was studied in Sprague-Dawley rats which indicated that there were no significant dose related differences in their body weight of exposed groups. Table 9 shows the anthropometric measurements of workers employed in jewellery units

Table 9
Anthropometric Measurements of the Selected Jewellery Unit Workers

Anthropometrics	Control group (A) (n=53)	Exposed groups		p Value ($p < 0.05$)
		Occasional (B) (n=85)	Frequent (C) (n=73)	
Height (cm) [§]	156 (154 - 161)	157 (154 - 162)	156 (153-160)	0.208 [¥]
Weight (Kg) [§]	65 (60 - 75)	65 (58 - 75.5)	62 (56-71)	0.115 [¥]
BMI (Kg/m ²) [§]	26.64 (23.9 - 30.4)	25.8 (23.4 - 29)	24.7 (22.9 - 27.9)	0.075 [¥]
Systolic pressure [§]	120 (114 - 120)	121 (115 - 130)	123 (115 - 130)	0.004[¥] ⚙️

Diastolic pressure*	74.94 ± 8.1	78.32 ±10.43	78.20 ± 8.17	0.074
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§ **Median value (Inter quartile range)**

* **Mean value ± standard deviation**

‡ **Kruskal Wallis test computed for groups with unequal sized samples and Mann Witney test computed between the groups**

ANOVA computed for groups with unequal sized samples and post hoc computed between the groups

© **The median difference is significant at 0.05 level.**

In the present study, there was no significant difference in body mass index (BMI) between exposed and control group workers ($p > 0.05$). Among the exposed groups, there was no significant difference ($p > 0.05$) in BMI between occasional (Group B) and frequent workers (Group C).

According to Kim *et al.* (2008) the oral toxicity of silver nanoparticles was studied in male and female Sprague-Dawley rats in which the rats did not show any significant changes in their body weight.

Systolic blood pressure was significantly increased ($p < 0.05$) in jewellery unit workers of B and C groups compared to the control (group A). The increase in systolic blood pressure in the studied population might indicate the insufficient sleep in subjects doing extensive overtime work or due to an increase in reactive oxygen species (ROS). Increase in systolic blood pressure might be due to insufficient sleep of the workers which is supported by the findings of Tochikubo *et al.* (1996). However, additional objective measures are warranted in order to come to a more reliable conclusion.

There was no significant difference ($p > 0.05$) in diastolic blood pressure between the exposed group workers and control group.

4.1.7 Levels of silver and metallothioneins (MTs) in serum among the jewellery unit workers

Median levels of silver and metallothioneins in the serum of control and jewellery unit workers are shown in Figure 7.

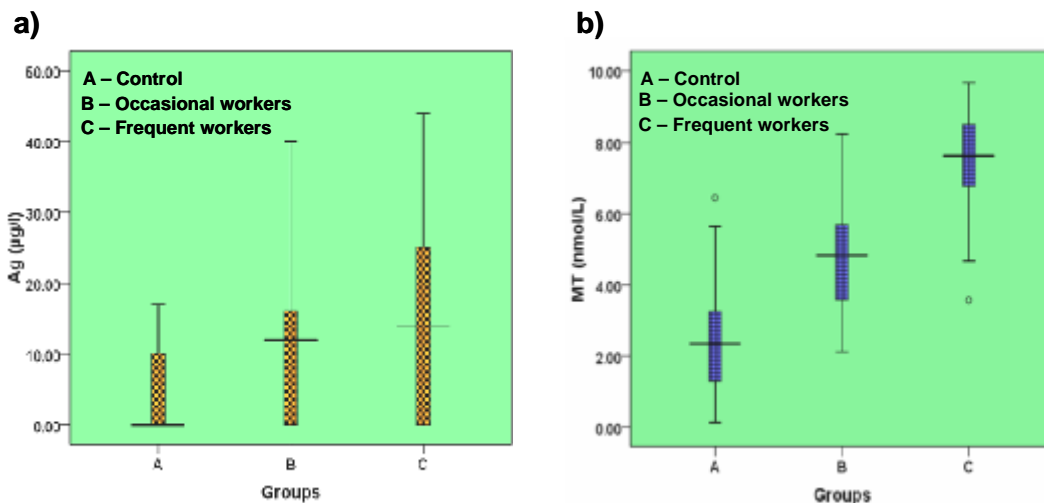
a) Silver

Pure gold is alloyed with copper, zinc, cadmium and silver in varying proportions to produce a wide range of karat gold. The levels of copper, zinc, cadmium and silver were assessed in the pooled sera samples of exposed and control group workers of the study. Copper and zinc were detected up to 0.1 ppm and 0.9 ppm respectively. Cadmium was not detected and silver was detected upto 0.08 ppm.

Workers of jewellery units had been exposed to hazardous compounds while processing gold in which silver is used in higher concentration. Silver is considered as xenobiotic compound with less known trace metal value in the human body. Therefore, the study was aimed to concentrate on silver exposure of jewellery unit workers who have been exposed to this metal daily over an extended period of time.

Figure 7

Serum Silver and Metallothionein Levels among Workers in Jewellery Units



Several case studies of silver exposure showed that small doses of silver compounds taken as medication over several months might CAUSE argyria in some humans. People who are working in silver compounds manufacturing factories inhale these compounds that may cause argyria. It may be due to the elevated level of silver in the air or duration of exposure at work place. It is also not known, what level of silver causes breathing problems, lung and throat irritation, or stomach pain in people (Agency for Toxic Substances and Disease Registry, 1990).

The level of silver in whole blood, plasma, serum or urine can be measured to know the health status and monitor the safety of exposed workers (Baselt, 2008). In the present study, level of serum silver was estimated in the jewellery unit workers.

Figure 7a shows that serum silver level was significantly raised ($p < 0.05$) in exposed groups (B and C) than control group (A). Among the exposed groups level of silver was significantly increased ($p < 0.05$) in group C compared to group B. Raised level of silver in serum of jewellery unit workers could be due to exposure to fumes and dusts of silver emitted during the manufacturing process of gold.

Armitage *et al.* (1996) studied the uptake of silver in the blood of workers occupationally exposed to silver that showed the blood silver level ranging from 0.1 to 23.0 $\mu\text{g/L}$ in which highest level of silver in blood was observed in reclaimers.

According to Eisses and Kaplan (2005), copper trafficking protein (ctr-1) can effectively transport silver into the cells exposed to even low micro molar concentration. $^{110}\text{AgNO}_3$ injected rats showed that silver metabolism resembles that of copper with respect to tissue distribution. Silver does not significantly inhibit internal copper absorption but it affects copper metabolism as reported by Hanson *et al.* (2001). Estimation of silver level in the biological samples may help to characterize the individual degree of exposure and the occurrence of adverse consequences.

Similar to the present study, several researchers reported that increased level of silver in blood, serum and plasma of workers might be due to the occupational exposure of silver. Industrial workers are exposed to silver compounds from occupational settings namely jewellery and silver reclamation (Armitage *et al.*, 1996), silver refineries (Williams, 1999) and soluble and insoluble silver plant (Wolbling *et al.*, 1988; Williams and Gardener, 1995; Di-Vincenzo *et al.*, 1985).

In the present study, the air silver level was not tested in the sampling site. It is probable that the workers were exposed to silver fumes and dusts arising from the operations involved in the jewellery manufacturing processes. The Occupational Safety and Health Administration (OSHA), Mine Safety and Health Administration (MSHA) and

the National Institute for Occupational Safety and Health (NIOSH) established a Recommended Exposure Limit (REL) of 0.01 mg/m³ for soluble, insoluble silver compounds and silver metal dusts (Occupational Safety and Health Administration, 1989; National Institute for Occupational Safety and Health, 2003).

b) Metallothioneins (MTs)

MTs are ubiquitous, low molecular weight proteins with the ability to bind heavy metals. They are rich in cysteine residues which are responsible for the formation of metal-thiolate cluster. However, their biochemical characterization and *in vivo* utilities remain mysterious. Multiple gene copies of MTs in humans indicate their involvement in cellular function, metal homeostasis and defense against toxicity of heavy metals (Egli *et al.*, 2006).

Most of the absorbed heavy metals primarily bind to metalloproteins such as metallothioneins. These metal-protein complexes are metabolized in liver and mostly excreted via urine and feces (Baldi *et al.*, 1988). Silver metabolism is modulated by induction and binding to metallothioneins. This complex mitigates the cellular toxicity of silver and contributes to tissue repair (Lansdown, 2006).

In the present study, level of serum MTs was significantly elevated ($p < 0.05$) in exposed groups (B and C) than control group A. Among exposed groups, MT activity was significantly raised ($p < 0.05$) in frequent workers (Group C) than occasional workers (Group B) as presented in Figure 7b.

Chang *et al.* (2006) revealed that the level of MT in peripheral blood lymphocytes of cadmium exposed workers was significantly higher than control group. Active biosynthesis of MTs was observed in the kidneys and liver of cadmium exposed animals as observed by earlier researchers.

A study on cadmium exposure and MT expression in smelter workers indicated a significant increase of MTs in the urine of exposed workers than controls and a positive association was observed between MTs and cadmium (Shaikh *et al.*, 1990a). Urinary MT level was increased in environmentally cadmium exposed Japanese population which led to renal dysfunction as opined by Shaikh *et al.* (1990b).

A recent animal study showed increased hepatic and decreased urinary MTs level after cessation of oral exposure to cadmium (Liang *et al.*, 2010). A study on administration of CdCl₂ and AgNO₃ in mice showed that both the salts enhanced the synthesis of MTs and also it served as an effective scavenger of reactive oxygen species (Srivastava *et al.*, 1995).

4.1.8 Hematological indices in jewellery unit workers

Red blood cell indices

Table 10 presents the levels of red blood cell indices among workers employed in jewellery units

Table 10

Levels of Red Blood Cell Indices among Jewellery Unit Workers

Red Blood Cell Indices	Control group (A) (n=53)	Exposed groups	
		Occasional workers (B) (n=85)	Frequent workers (C) (n=73)
RBC [§] (10 ⁶ /µl)	4.97 (4.7- 5.29)	4.8 (4.6 - 5.05) ^a	4.6 (4.28-4.94) ^{a,b}
Hb* (g/dl)	14.58 ± 1.27	13.73 ± 1.24 ^a	13.54 ± 1.38 ^a
HCT* (%)	44.53 ± 3.48	41.82 ± 4.11 ^a	40.61 ± 3.90 ^a
MCH [§] (pg)	28.9 (27.75 - 30)	30.0 (28.2 - 30.85)	29.7 (28.4 - 31.3) ^a
MCHC [§] (g/dl)	32.6 (31.7 - 33.2)	33.3 (32.1- 36) ^a	35.0 (32.3 - 36) ^a
RDW [§] (fl)	44.2 (41.8 - 46.2)	44.1 (43 - 46.9)	45 (43.4 - 47)
MCV [§] (fl)	88.6 (85.9 - 91.5)	87 (84 - 89.8)	87.7 (82 - 93.7)

[§] Median value (Inter quartile range)

Kruskal Wallis test computed for groups with unequal sized samples and Mann Witney test computed between the groups

*** Mean value \pm standard deviation**

ANOVA computed for groups with unequal sized samples and post hoc computed between the groups

^a Statistically significant ($p<0.05$) compared to control

^b Statistically significant ($p<0.05$) compared to occasional workers

Red blood cell (RBC) count was significantly decreased ($p<0.05$) among exposed group workers compared to control group. Among the exposed groups, RBC count was significantly decreased ($p<0.05$) in frequent workers (Group C) compared to occasional workers (Group B). Significant decrease was observed ($p<0.05$) in hemoglobin (Hb) and hematocrit (HCT) levels of exposed workers (Group B and C) compared to control group (A). But HCT and Hb did not show any significant difference among the exposed groups.

A similar result has been reported by Cheraghi *et al.* (2014) in which RBC and hemoglobin levels were decreased considerably in male and female mice of silver nanoparticle treated groups than control group.

According to Shaluei *et al.* (2013), RBC count, Hb and HCT levels were significantly reduced in silver nanoparticle treated silver carp than control group.

The results of the present study are supported by the reduction in Hb level observed in oil refinery workers (Yartireh and Hashemian, 2013), lead furnace workers and pellet handlers (Bashir *et al.*, 1995).

The red cell indices namely mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) levels were significantly increased ($p<0.05$) in frequent workers of exposed group than the controls. MCHC alone was significantly increased ($p<0.05$) in occasional workers than the controls. But levels of MCHC and MCH did not show significant differences among the two exposed groups (Group B and C). An increase in MCH and MCHC in the present study might be due to exposure to pollutants in the jewellery units.

Mean corpuscular volume (MCV) and red blood cell distribution width (RDW) did not show any significant difference ($p>0.05$) between jewellery unit workers and control group of the study. Lee *et al.* (2013) reported that the hematological parameters such as MCV and RDW did not show significant changes after 28 days of silver nanoparticle administration to Sprague Dawley rats.

Total, differential leucocyte and platelet count

Table 11 illustrates the total, differential leucocyte and platelet count in the blood of workers employed in the jewellery units.

White blood cells (WBC) or leucocytes are a part of the immune system. The type of leucocytes normally found in blood are lymphocytes, granulocytes (neutrophils, eosinophils and basophils) and monocytes (Chan *et al.*, 2010). Therefore in the present study, measurement of blood leucocytes in the jewellery unit workers was found to be imperative to assess the influence of silver on immunological responses.

Table 11
Total, Differential Leucocyte and Platelet Count in Blood among Jewellery Unit Workers

Hematological Indices	Control group (A) (n=53)	Exposed groups	
		Occasional workers (B) (n=85)	Frequent workers (C) (n=73)
WBC [§] (10^3 / μ l)	6.9 (5.8 - 7.6)	7.0 (6.1 - 8.0)	7.8 (6.5 - 8.5) ^{a,b}
Lymphocytes [§] (10^3 / μ l)	2.6 (2.2 - 2.95)	2.8 (2.2 - 3.5)	3.0 (2.5 - 4.1) ^{a,b}
Mixed cells [§] (10^3 / μ l)	0.8 (0.6 - 1.0)	0.9 (0.7 - 1.1) ^a	1.0 (0.8 - 1.2) ^a
Neutrophils [§] (10^3 / μ l)	3.6 (3.1 - 4.2)	3.5 (3.1 - 4.0)	3.3 (2.8 - 4.0)
Platelet* (10^3 / μ l)	231.6 \pm 51.5	247.4 \pm 58.6	246.4 \pm 53.8
PDW [§] (fl)	11.5 (10.7 - 12.3)	11.5 (10.2 - 13.5)	10.4 (9.2 - 12) ^{a,b}
MPV [§] (fl)	9.5 (9.0 - 9.8)	9.4 (8.7 - 10.8)	9.1 (8.1 - 10.1) ^{a,b}

[§] Median value (Inter quartile range)

Kruskal Wallis test computed for groups with unequal sized samples and Mann Witney test computed between the groups

*** Mean value \pm standard deviation**

ANOVA computed for groups with unequal sized samples and post hoc computed between the groups.

^a Statistically significant ($p < 0.05$) compared to control

^b Statistically significant ($p < 0.05$) compared to occasional workers

It is evident from table 11 that the total leucocyte count was significantly raised ($p < 0.05$) in exposed groups C in contrast to control group (A). Among the exposed groups, WBC count was significantly increased ($p < 0.05$) in frequent workers than occasional workers. Differential count namely lymphocytes and mixed cells (including basophils, eosinophils and monocytes) were significantly increased ($p < 0.05$) in exposed groups (Group B and C) than the control group (Group A). Among the exposed groups, lymphocyte count was significantly raised ($p < 0.05$) in group C when compared to group B. But mixed cell count did not show the significant differences among the two exposed groups (Group B and C).

WBC and Lymphocytes counts were increased in exposed groups of the study which indicated resistance or synthesis of metalloprotein against silver. According to Bao *et al.* (2012) low dose of cadmium (0.1mg CdCl₂ / kg) did not show any toxic effects on rat peripheral blood lymphocytes and WBC. But it induced the expression of metallothionein in bone marrow cells.

The mixed cell count such as basophils, eosinophils and monocytes were significantly increased ($p < 0.05$) in exposed groups than control group, but prediction of individual changes in each cell type could not be possible due to lack of information provided by the three part hematological analyzer used in the study. According to Sharma and Langer, (2014), an increase in number of differential leucocyte count (monocytes and eosinophils) was observed in fish (*Garra gotyla gotyla*) for 9 weeks of experimental duration by the effect of manganese.

Platelet indices are potentially useful markers for the early diagnosis of thromboembolic diseases. An increase in both mean platelet volume (MPV) and platelet

distribution width (PDW) due to platelet activation, resulting from platelet swelling and pseudopodia formation were hypothesized (Vagdatli *et al.*, 2010).

In the present study, number of neutrophils and platelets did not show any significant difference ($p>0.05$) between control and exposed groups. Similar result was observed by Kianoush *et al.* (2013) in lead exposed workers. Divya priya and Suja (2012) reported no significant difference in the neutrophils count between cement dust exposed workers and controls.

The study also showed that PDW and MPV levels decreased significantly ($p<0.05$) in exposed groups (C) than control group (A). Similarly among the exposed groups, their levels were significantly decreased ($p<0.05$) in group C when compared to group B. MPV is a marker of platelet activation that involves in the pathophysiology of a variety of pro-inflammatory diseases (Wang *et al.*, 2013). The significant decrease in MPV may be due to disruption in bone marrow during the synthesis of platelets (Abdelhalim and Moussa, 2012).

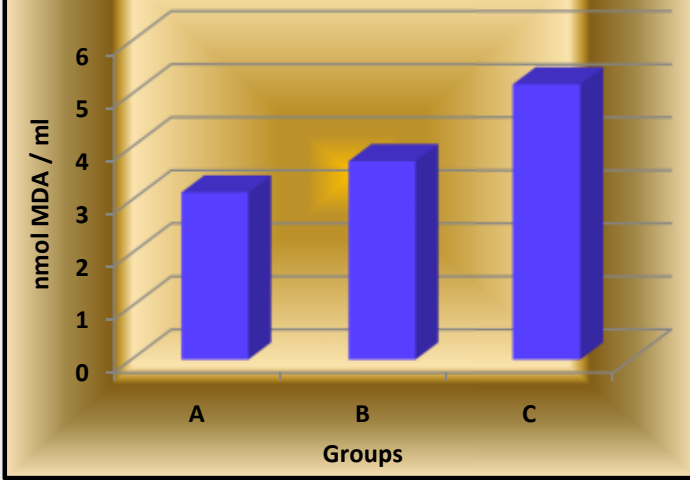
From the study, though MPV and PDW were significantly decreased in exposed groups than control group, since the platelet count is normal, a low MPV and PDW do not mean much, because the size of the normal mature platelet is small.

The results of the present study revealed some of the hematological values to be associated with silver absorption which is supported by the findings of Hogstrand *et al.* (1999) and Vlachou *et al.* (2007).

4.1.9 Level of lipid peroxidation in the serum of the chosen population

Figure 8 presents the mean level of thiobarbituric acid reactive substances in the serum of jewellery unit workers

Figure 8. Mean Level of Serum Thiobarbituric Acid Reactive Substances among Jewellery Unit Workers



A – Control, B – Occasional workers, C – Frequent workers

Free radicals and oxidative stress generate the lipid peroxidation process in an organism. Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids peroxidation in the cells. An increase in free radicals causes overproduction of MDA (Gawel *et al.*, 2004).

Elevated level of MDA in the serum has been documented in some common diseases. Moreover, previous reports showed that the peroxidative damage could be caused by nano particles (Potter *et al.*, 2011). In the present study, mean level of MDA was significantly increased ($p < 0.05$) in the serum of jewellery unit workers (Group B and C) than control (Group A). Among the exposed workers, level of MDA was significantly increased ($p < 0.05$) in group C compared to group B which could be attributed to exposure to silver fumes and dusts for a long period.

An increase in MDA level was noticed in gills and digestive gland of mussels (*Mytilus edulis*) exposed for 21 days to either cadmium, silver or mercury (Geret *et al.*, 2002). Liao *et al.* (2006) reported that mean plasma MDA level in the electronic industry workers was significantly higher than in referents. Also the level of MDA in the exposed workers was positively correlated with the levels of urinary gallium and arsenic. It was suggested that MDA can be used as an indicator of lipid peroxidation and it could be stimulated by gallium and arsenic exposure.

According to Babu *et al.* (2006), a significant increase in plasma lipid peroxidation product could be used as biomarker of oxidative stress in cadmium exposed workers. A significant increase in lipid peroxidation products was observed in

carbon exposed population than controls which increased as its exposure period increased (Reddy *et al.*, 1998).

4.1.10 Liver function markers among the jewellery unit workers

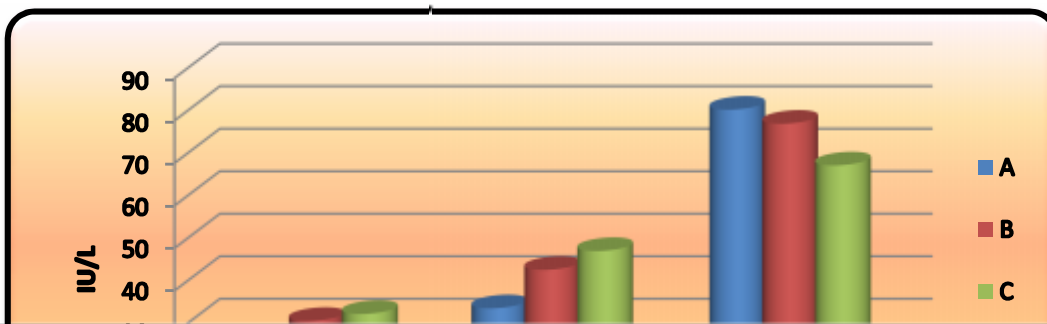
Liver and kidney are important organs for detoxification and excretion of xenobiotics. Silver is absorbed into the human body and enters into the systemic circulation as a protein complex that has to be eliminated through the liver and kidneys (Lansdown, 2006).

Liver performs many biochemical functions. The liver cells are rich in enzymes that convert biologically active substances such as poisons, hormones and drugs into less harmful by products (Garrett and Grisham, 2013). Liver injury induced by metals and chemicals were identified by many researchers and the highest accumulation of the metals was recorded in the liver (Mohamed and Gad, 2008). A number of serum hepatic enzymes are used as diagnostic markers of hepatic damage.

The liver function in jewellery unit workers was evaluated by estimating the activities of enzymes in serum namely alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP). Mean activities of these enzymes are shown in Figure 9.

Figure 9

Mean activities of Serum Alanine amino transferase (ALT), Aspartate amino transferase (AST) and Alkaline phosphatase (ALP) among Jewellery Unit Workers



Serum AST and ALT activities were found to be increased significantly ($p < 0.05$) in jewellery unit workers (Group B and C) compared to controls (Group A) of the study. Among the exposed workers, activities of these liver enzyme markers were found to be increased in frequent workers (Group C) than occasional workers (Group B).

Activities of ALT and AST were increased in cadmium exposed rats studied by Kara *et al.* (2005). They postulated that metallothioneins can act as antioxidant against cadmium toxicity and lipid peroxidation. Also a survey on Egyptian tilapia fish farms revealed high concentration of heavy metals in water and fish tissues which altered the liver enzymes, serum proteins and MT gene expression as reported by Abumourad *et al.* (2013).

The transaminases activities might serve as liver function tests which might indicate any inflammation in the hepatic cells by silver exposure and also specify the silver induced hepatocellular damage.

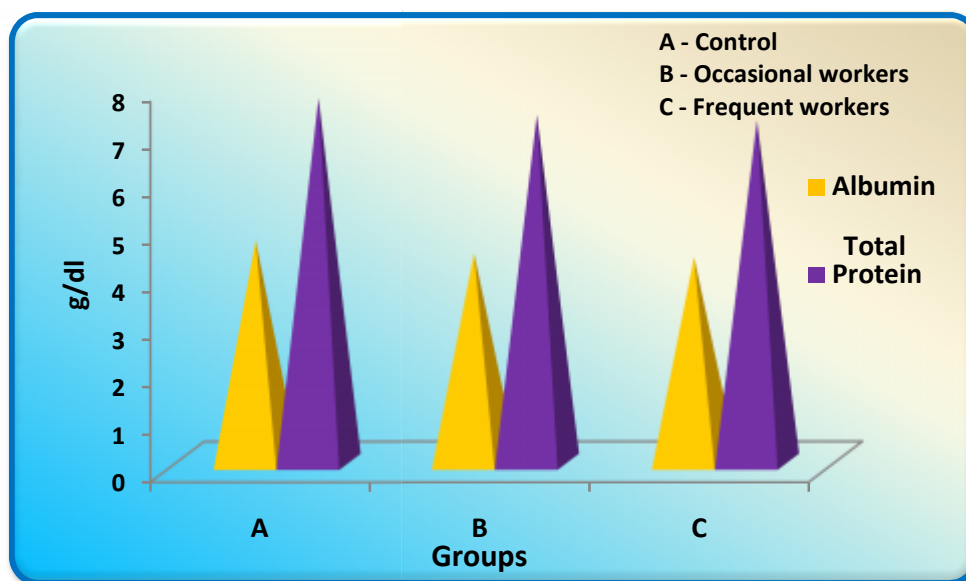
The AST/ALT ratio is occasionally used to differentiate the causes of liver damage (hepatotoxicity). In the present study, mean and standard deviation of AST/ALT ratio among control (Group A), occasional workers (Group B) and frequent workers (Group C) were observed as 0.76 ± 0.23 , 0.72 ± 0.24 and 0.69 ± 0.19 respectively. AST/ALT ratio did not show any significant difference ($p > 0.05$) between jewellery unit workers (Group B and C) and controls (Group A).

Serum alkaline phosphatase (ALP) activity was found to be decreased significantly ($p < 0.05$) in the jewellery unit workers (Group B and C) compared to control group (Group A). Among the jewellery unit workers, ALP activity was decreased significantly ($p < 0.05$) in group C (Frequent workers) than group B (Occasional workers) which might be due to the inhibition of ALP by silver which is supported by the findings of Chen *et al.* (2000).

Figure 10 presents the mean levels of albumin and total protein in the serum of jewellery unit workers.

Figure 10

Mean Levels of Albumin and Total Protein in Serum among Jewellery Unit Workers



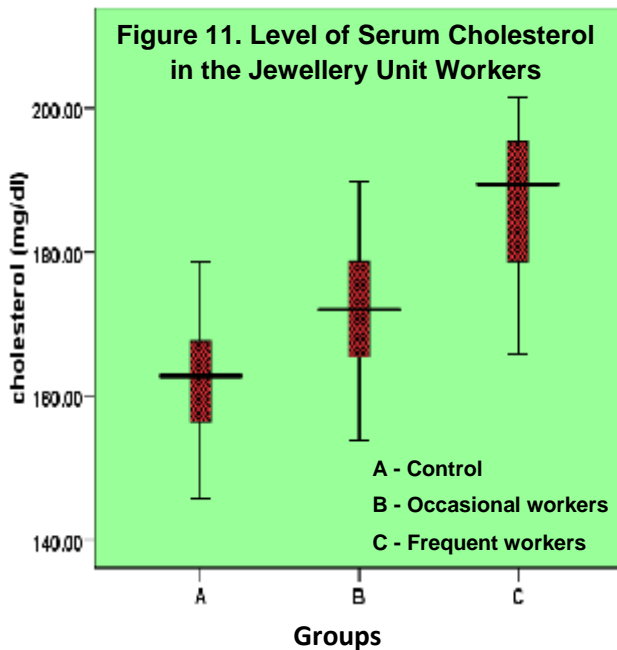
Another measurable liver function is reflected in the total protein and albumin levels, which are the markers of liver biosynthetic capacity.

In the present study, the levels of serum total protein and albumin were found to be decreased significantly ($p < 0.05$) in exposed workers (Group B and C) than control group workers (Group A). Among the jewellery unit workers, these indices did not show any significant difference ($p > 0.05$) between frequent workers (Group C) and occasional workers (Group B). Similar findings were observed by Saadat and Ansari-Lari (2005) and Patil *et al.* (2007) among gasoline exposed filling station workers.

It was also demonstrated that there was a decrease in serum albumin and total protein on oral exposure to nitrocellulose thinner in male rats (Uboh and Ufot, 2013).

Serum total cholesterol level among jewellery unit workers

The median level of serum total cholesterol of jewellery unit workers is depicted in Figure 11.



Liver plays a central role in the regulation of lipid metabolism.

In the present study, level of serum cholesterol was significantly increased ($p < 0.05$) in the jewellery unit workers (Group B and C) than controls (Group A). Among the exposed workers, level of total cholesterol was significantly increased ($p < 0.05$) in group C compared to group B.

A study conducted by Oner *et al.* (2008) reported that increased level of total cholesterol in serum was recorded in freshwater fish (*Oreochromis niloticus*) exposed to low level (0.05 mg/L) of silver metal.

Vinodhini and Narayanan (2009) observed elevated level of total cholesterol in common carp (*Cyprinus carpio L.*) on exposure to heavy metal pollutants. Larregle *et al.* (2008) observed that cadmium exposure altered directly or indirectly the serum lipid content and liver lipid metabolism in adult male Wistar rats.

It was found that there was a significant increase in total cholesterol levels of nickel treated rats (Das *et al.*, 2006). Occupational exposure to lead in various craftman

in Nigeria was studied by Ademuyiwa *et al.* (2005) who reported that their total cholesterol level was twice higher than that of controls.

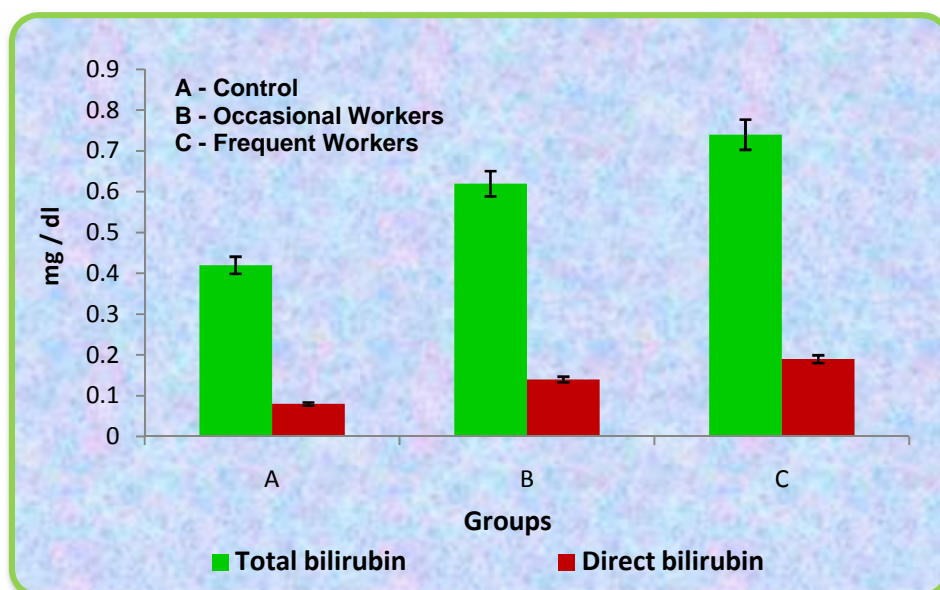
In the present study, silver exposure seemed to modify the metabolism of lipids increasing the serum cholesterol level which is supported by Elle *et al.* (2013) who showed that exposure to 500 mg/kg of silver nanoparticles in Sprague Dawley rats resulted in liver damage by the dysfunction of lipid metabolism.

Serum total and direct bilirubin among jewellery unit workers

Mean levels of serum total and direct bilirubin among jewellery unit workers are shown in Figure 12.

Figure 12

Serum Total and Direct Bilirubin Levels among Jewellery Unit Workers



In the present study, serum total and direct bilirubin levels in the jewellery unit workers (Group B and C) were found to be increased significantly ($p < 0.05$) compared to control group (Group A). Among the exposed groups, the levels of total and direct bilirubin were raised significantly ($p < 0.05$) in group C (Frequent workers) than group B (Occasional workers). Occupational and environmental metal exposure were found to be associated with significant changes in hepatic clearance of bilirubin as opined by Shebl and Sarhan (2008).

The result of the present study could be supported by the work of Brodtkin *et al.* (2001). They showed that mean direct bilirubin concentration and direct / total bilirubin ratio were increased in styrene exposed workers compared to control. They also demonstrated that direct bilirubin concentration and direct / total bilirubin ratio were associated with exposure to styrene.

In the present study there was no significant difference ($p>0.05$) in serum glucose level among exposed and control group subjects.

4.1.11 Renal function parameters among the jewellery unit workers

Several heavy metals have been known as nephrotoxic to humans and animals. Renal lesions as a direct consequence of these toxic agents have been studied intensively. Occupational exposure to silver compounds may produce toxic effect on renal function as reported by Drake and Hazelwood (2005).

Research on experimental animals indicated that MTs can act as a carrier molecule for metals such as cadmium from liver to kidney. Cadmium – MTs complex would be filtered through glomerulus and reabsorbed by proximal tubules. MTs absorption in the tubules, their catabolism and synthesis must play an important role for the development and progress of the tubular dysfunction (Falck *et al.*, 1983).

Hence, kidney function in jewellery unit workers was assessed by estimating the levels of serum creatinine, urea and uric acid and levels of urine total protein, creatinine and total protein to creatinine ratio (P/C ratio). The median values of these biochemical parameters in the jewellery unit workers are presented in Table 12.

Table 12
Serum and Urine Renal Function Parameters among Jewellery Unit Workers

Renal Function Parameters [†] (mg / dl)	Controls	Exposed groups	
		Occasional workers	Frequent workers
Serum	22 (19.12 - 29.6)	28.8 (24.79 - 35.83) ^a	48 (42.7 - 54) ^{a,b}

Urea	4.53 (3.9 - 5.2)	5.9 (5.15 –7.0) ^a	9.5 (8.58 - 9.8) ^{a,b}
Uric acid			
Creatinine	0.92 (0.82 - 0.99)	0.91 (0.80 - 0.97)	1.34 (1.23 - 1.57) ^{a,b}
Urine			
Total Protein	0.13 (0.00 - 0.71)	4.56 (2.24 - 8.18) ^a	6.0 (2.62 - 11.56) ^a
Creatinine	87 (71.0 - 167)	78 (63.0 - 87) ^a	78 (64.0 - 87) ^a
Total Protein to Creatinine ratio (P/C) (mg/mg)	0.02 (0.00 - 0.06)	0.07 (0.03 - 0.11) ^a	0.09 (0.04 - 0.16) ^a

[†] Median value (Inter quartile range)

Kruskal Wallis test computed for groups with unequal sized samples and Mann Witney test computed between the groups

a Statistically significant (p<0.05) compared to control

b Statistically significant (p<0.05) compared to occasional workers

The serum urea and uric acid levels in the jewellery unit workers (Group B and C) were found to be increased significantly (p<0.05) compared to controls (Group A). Among the exposed groups, these parameters were elevated significantly (p<0.05) in frequent workers (Group C) compared to occasional workers (Group B). The level of serum creatinine in frequent workers (Group C) was increased significantly (p<0.05) than control group (Group A) and occasional workers (Group B). But there was no significant changes (p>0.05) noticed between controls (Group A) and occasional workers (Group B). The increased levels of these parameters in serum might suggest minor renal impairment in exposed workers.

Occupational lead exposure significantly increased the serum uric acid level (p<0.01) of petroleum products exposed workers compared to unexposed workers as reported by Dioka *et al.* (2004). The result of the present study could be supported by Ahmed *et al.* (2008) who reported a significant increase in serum uric acid in lead smelters compared to reference group. As per the report of Mason *et al.* (1999), a considerable increase in plasma creatinine level was seen in welding workers using cadmium containing silver solder than the control group. Silver nanoparticles seemed to

cause significant increase in blood urea nitrogen and creatinine levels in male laboratory mice compared to control group (Dayani *et al.*, 2014).

Proteinuria is an early sensitive and reliable marker of renal damage. Measurement of protein in 24 hours urine is not convenient and is subject to collection errors. Several studies have made use of protein/creatinine (P/C) ratio of random urine samples in different clinical settings to estimate 24 hours proteinuria. They found out P/C ratio as an accurate test to define critical level of proteinuria (Shaw *et al.*, 1983; Gaspari *et al.*, 2006; Antunes *et al.*, 2008).

Mason *et al.* (1999) reported that urinary retinol binding protein, total protein and albumin levels were significantly increased in soldering workers. From urinary protein measurements, there was an evidence of increasing renal tubular damage in workers who had been brazing for several years with solders containing cadmium. A similar result was observed in the present study where the levels of P/C ratio in the jewellery unit workers (Group B and C) were found to be increased significantly ($p < 0.05$) compared to controls (Group A). Among the exposed groups, there was no significant difference in the levels of P/C ratio ($p > 0.05$) between frequent workers (Group C) and occasional workers (Group B).

Protein/Creatinine ratio (P/C) of less than 0.2 mg/mg can be considered as within the range of normal limits described by Abuelo (1983). But low level of proteinuria identified as urine protein/ creatinine ratio of 0.11 mg/mg or higher is associated with substantial excess mortality risk in healthy adults, even after exclusion of diabetics and those with reduced kidney function as reported by Fulks *et al.* (2012).

In the present study median level of P/C ratio was well within the range of normal limit. But it was similar to the reports of Fulks *et al.* (2012).

Kidneys are the primary target organs that take up and accumulate silver from blood. The accumulation of this metal in the kidneys is very rapid. The segments of the proximal tubules in the kidneys are the principal portions of the nephrons that take up and accumulate silver.

Silver nanoparticles (Ag-NPs) were found to accumulate in the basement membranes of the renal tubules in the middle and terminal parts of outer and inner medulla and in the cortex. Also Ag-NPs were detected in the nuclei and cytoplasm of cells in inner medulla of the kidney (Kim *et al.*, 2009).

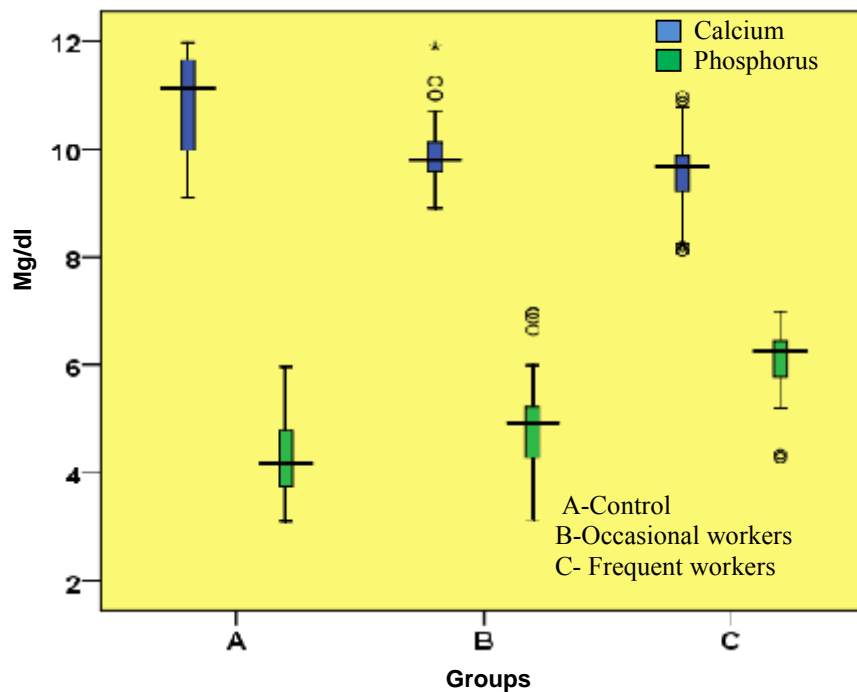
Franchini *et al.* (2005) opined that nephrotoxic properties of silver may arise mainly from the tubular reabsorption of silver –protein complexes and start a cascade of events leading to cell membrane damage and oxidative stress.

4.1.12 Serum mineral status in jewellery unit workers

The median levels of calcium and phosphorus in serum among the jewellery unit workers are shown in Figure 13.

Figure 13

Serum Calcium and Phosphorus Levels of Workers Employed in Jewellery Units



The level of calcium in the serum was found to be decreased significantly whereas the level of phosphorus was increased significantly ($p < 0.05$) in the jewellery unit workers (Group B and C) compared to control (Group A). Among the exposed

groups, the levels of calcium was decreased and phosphorus was increased significantly ($p < 0.05$) in frequent workers (Group C) than occasional workers (Group B).

The result of the present study could be supported by the findings of Jarup *et al.* (2000) in which a group of cadmium workers showed evidence of a multiple tubular reabsorptive defect of tubular proteinuria with increased urinary excretion of calcium and low level of serum calcium.

Jarup *et al.* (1998) opined that the initial sign of cadmium induced renal lesions are tubular proteinuria followed by decreased glomerular filtration rate. Also secondary effect of cadmium exposure was found to be impairment of calcium metabolism.

There are several potential mechanisms that may explain the association of serum phosphorus levels with greater risk of cardio vascular diseases. High phosphorus levels were known to inhibit 1, 25-dihydroxyvitamin D synthesis in some studies. Lower levels of 1,25-dihydroxyvitamin D were hypothesized to decrease cardiac contractility and to increase coronary calcification which revealed that higher serum phosphorus levels could contribute to a greater vascular risk (Dhingra *et al.*, 2007).

4.1.13 Association between median levels of serum silver and reported clinical symptoms among jewellery unit workers

Table 13 shows the non parametric analysis ie., Chi square and Fisher's exact test between median levels of serum silver and reported clinical symptoms among jewellery unit workers to find any association between them.

Serum silver level in jewellery unit workers was higher ($p < 0.05$) than unexposed workers. An association between serum silver level and some of the reported symptoms of workers in the study was observed.

Table 13

Association between Median Level of Serum Silver and Clinical Symptoms among the Jewellery Unit Workers (n = 158)

Clinical Symptoms	Number	Percentage	Median Levels of Silver (µg/L)		p Value
			< 20	>20	
Throat pain					
Yes			18	11	0.996[§]
No	29	18.35			
	129	81.65	79	50	
Nausea					
Yes			5	3	1.000*
No	8	5.06			
	150	94.94	92	58	
Abdominal pain					
Yes			30	37	0.000[§]
No	67	42.41			
	91	57.59	67	24	
Headache					
Yes			29	8	0.012
No	37	23.42			
	121	76.58	68	53	
Dry skin					
Yes			13	12	0.876[§]
No	25	15.82			
	133	84.18	84	49	
Dizziness					
Yes			15	10	0.876[§]
No	7	4.43			
	151	95.57	82	51	
Eye irritation					
Yes			13	16	0.043[§]
No	29	18.35			
	129	81.65	84	45	
Muscle weakness					
Yes			23	28	0.007[§]
No	51	32.28			

	107	67.72	74	33	
Wheezing					
Yes					
No	3	2	1	2	0.560*
	155	98	96	59	
Redness of skin					
Yes					
No	7	4.43	2	5	0.068*
	151	95.57	95	56	
Decreased appetite					
Yes					
No	27	17.09	18	9	0.536[§]
	131	82.91	79	52	
Absolutely normal					
Yes					
No	48	30.38	25	23	0.089[§]
	110	69.62	72	38	

p < 0.05 significant * Fisher's exact test [§] Chi square test

Prevalence of symptoms such as abdominal pain (p = 0.000), headache (p = 0.012), eye irritation (p = 0.043) and muscle weakness (p = 0.007) among jewellery unit workers were significantly related to the level of serum silver. Other reported symptoms such as throat pain, nausea, dry skin, dizziness, wheezing, redness of the skin and decreased appetite of the workers were not significantly associated (p > 0.05) with serum silver level.

Soluble silver compounds are readily absorbed and have the potential to produce adverse health effect on the human body such as decreased blood pressure, diarrhoea and stomach irritation. Chronic symptoms from prolonged intake of low doses of silver salts were found to accumulate in the skin, liver, kidneys, corneas, gingiva, mucous membranes, nails and spleen (Sue *et al.*, 2001). Greater occupational risk may be experienced by those exposed to inhalation of airborne silver particles. These

nanocrystalline particles were found to dissolve more rapidly in moisture and achieve greater absorption leading to greater lung volumes (Burrell, 2003).

Lansdown (2010) proved that inhalation of soluble silver compounds caused both upper and lower respiratory tract irritation. Bronchitis, emphysema and reduction in pulmonary volume were observed when industrial workers were exposed to metallic silver as well as to other metals. Also, inhalation of silver compounds were found to cause flu-like symptoms with metallic taste, cough, chest pain, muscle weakness or pain and increased white blood cell count.

Respiratory irritation, eye irritation, sneezing, stuffy or runny nose and sore throat were noticed in silver and other metal powder manufacturing company workers as reported by Panyala *et al.* (2008). Skin contact with silver compounds were found to cause mild allergic reactions such as rashes, swelling and inflammation in some people.

4.1.14 Correlation between serum silver levels and selected parameters among jewellery unit workers

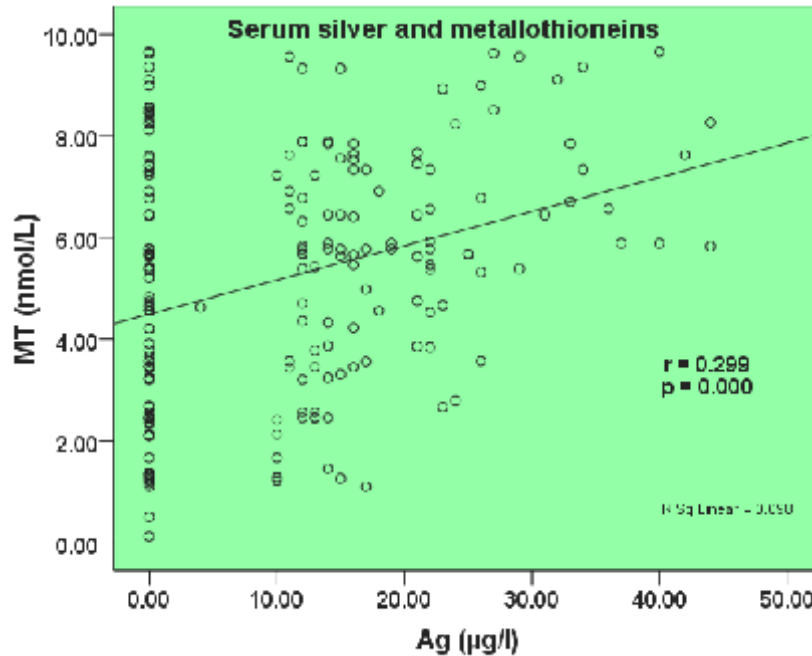
Diagnostic laboratories assess the functions of the organs through various biochemical parameters. Many biochemical parameters tend to have specificity for an organ and correlation analysis of these parameters might help to analyse the association among them. Correlation analysis was carried out between serum silver and selected parameters among the jewellery unit workers.

A significant positive correlation between serum silver and MTs ($r_s = 0.299$) indicated that silver is capable of inducing MTs which effectively immobilize and scavenge this metal. The level of MT was negatively correlated with red blood cells, hemoglobin and hematocrit ($r_s = -0.333$, $r_s = -0.244$ and $r_s = -0.296$ respectively) that might be due to the homeostasis of essential trace metals such as Cu, Fe and Zn by MTs. This result of the study could be supported by Wood *et al.* (2011) and Gulec and Collins (2013).

Figure 14 illustrates the correlation between levels of serum silver and metallothioneins among jewellery unit workers.

Figure 14

Correlation between Levels of Serum Silver and Metallothioneins among Jewellery Unit Workers



There was no correlation observed between lymphocytes, mixed cells and white blood cells ($r_s = 0.00$, $r_s = 0.02$ and $r_s = 0.12$ respectively) and serum silver in the present study. However a positive correlation of metallothioneins with WBC, mixed cells and lymphocytes ($r_s = 0.146$, $r_s = 0.178$ and $r_s = 0.257$ respectively) was noticed. This response is suggested to be dependent on the amount of absorbed dose of silver and also white series of cells which seemed to provoke an inflammatory response against it that is supported by the findings of Caciari *et al.* (2012).

Table 14 illustrates the correlation between serum silver and selected biochemical parameters among the jewellery unit workers.

Table 14

Correlation between Silver and Selected Parameters in the Jewellery Unit Workers

Variables	Silver	P/C	ALT	AST	T. Bilirubin	ALP
Silver	1.000	0.323**	0.420**	0.209**	0.255**	-0.294**

P/C	-	1.000	0.475**	0.213**	0.296**	-0.276**
ALT	-	-	1.000	0.220**	0.413**	-0.500**
AST	-	-	-	1.000	0.209**	-0.226**
T. Bilirubin	-	-	-	-	1.000	-0.244**
ALP	-	-	-	-	-	1.000

** Correlation is significant at 0.01 level

P/C ratio- Urine protein and creatinine ratio

ALT - Alanine amino transferase

AST - Aspartate amino transferase

T.Bilirubin - Total bilirubin

ALP - Alkaline phosphatase

In the present study, there were significant positive correlation between the levels of serum silver and urine protein and creatinine ratio ($r_s = 0.323$) which might be due to the critical effects of silver on kidney function as opined by Gonick (2008).

A significant positive correlation was noticed between ALT and AST activities and serum silver ($r_s = 0.420$, $r_s = 0.209$ respectively) which is supported by the view of Deng *et al.* (2013). They showed increased levels of AST and ALT in a population who had high occupational exposure to manganese. A significant negative correlation was noticed between serum silver ($r_s = - 0.294$) and ALP activity that was supported by the observation of Chen *et al.* (2000).

A significant positive correlation was noticed between levels of serum silver and total bilirubin ($r_s = 0.255$). This is supported by the findings of Brodtkin *et al.* (2001). They showed that mean direct bilirubin concentration and direct / total bilirubin ratio were increased in styrene exposed workers compared to control.

A positive correlation between ALT, AST and negative correlation of ALP activities and serum silver proved an indication of altered liver function.

4.1.15 Correlation between lipid peroxidation and selected biochemical parameters among jewellery unit workers

Correlation between lipid peroxidation and selected biochemical parameters among jewellery unit workers are presents in Table 15.

Table 15

Correlation between Lipid Peroxidation and Selected Biochemical Parameters among Jewellery Unit Workers

Variables	LP	Creatinine	Urea	Uric acid	P	Ag	MT
LP	1.000	-	-	-	-	-	-
Creatinine	0.313**	1.000	-	-	-	-	-
Urea	0.400**	0.635**	1.000	-	-	-	-
Uric acid	0.355**	0.587**	0.676**	1.000	-	-	-
P	0.395**	0.537**	0.511**	0.579**	1.000	-	-
Ag	0.212**	0.302**	0.307**	0.294**	0.310**	1.000	-
MT	0.426**	0.582**	0.700**	0.762**	0.595**	0.299**	1.000

** Correlation is significant at 0.01 level

LP – Lipid peroxidation

P – Phosphorus

Ag – Silver

MT - Metallothioneins

A significant positive correlation was observed between lipid peroxidation and creatinine, urea and uric acid. A significant increase in creatinine, lipid peroxidation and uric acid levels in the serum were observed in ethylene glycol administered rats as discussed by Kamboj *et al.* (2011). They also pointed that oxidative stress caused the destruction of cells and increased the levels of renal function parameters.

From the present study, a significant positive correlation was noticed between lipid peroxidation and serum phosphorus which might suggest that oxidative stress might alter the micronutrients absorption and transport in the body (Peraza *et al.*, 1998).

Lipid peroxidation was positively correlated with serum silver ($r_s = 0.212$) and serum MT ($r_s = 0.426$). Correlation analysis of the study might suggest age dependency and a protective effect of metallothioneins expression on silver exposure that might stimulate the lipid peroxidation. This fact is supported by the work of Liao *et al.* (2006) and Escorcía and Chang (2010).

4.1.16 Correlation between years of exposure to silver fumes and selected parameters among jewellery unit workers

Table 16 illustrates the correlation between years of exposure to silver fumes and selected parameters in the jewellery unit workers.

Years of exposure to silver fumes was positively correlated with Metallothioneins ($r_s = 0.406$). Renal tubular dysfunction and consequent MT protein in urine had been generally accepted as the main effect of long term low level exposure to cadmium (Hallenbeck, 1984; Kawada *et al.*, 1990). Silver metabolism is modulated by induction and binding to metallothioneins. This complex mitigates the cellular toxicity of silver (Lansdown, 2006).

A significant positive correlation between years of exposure to silver fumes and level of silver ($r_s = 0.231$) was observed. Urea, creatinine and cholesterol levels were increased when the years of exposure to silver fumes increased which could be supported by the report of Kim *et al.* (2009). They indicated that significant dose dependent changes were found in creatinine, urea and cholesterol levels of silver nanoparticle (125mg/kg) treated male and female rats.

Table 16

Correlation between Years of Exposure to Silver Fumes and Selected Parameters among Jewellery Unit Workers

Variables	Correlation coefficient (r_s)
Correlation between years of exposure to silver fumes and	
Metallothioneins	0.406**
Silver	0.231**
Urea	0.596**
Uric acid	0.475**
Creatinine	0.592**
Cholesterol	0.436**
ALP	-0.512**
ALT	0.345**
AST	0.175*
P/C ratio	0.152*

r_s Spearman correlation coefficient
** Correlation is significant at 0.01 level
* Correlation is significant at 0.05 level

ALP - Alkaline phosphatase
ALT - Alanine amino transferase
AST - Aspartate amino transferase
P/C ratio - Protein / Creatinine ratio

Serum uric acid level was found to be positively correlated with years of exposure to silver fumes which was supported by the study of Hadrup *et al.* (2012). They demonstrated that administration of nanoparticulate silver increased allantoin and uric acid excretion in Wistar rats.

A significant negative correlation was noticed between years of exposure to silver fumes ($r_s = -0.512$) and ALP activity and a significant positive correlation was observed between years of exposure to silver fumes and cholesterol, ALT and AST ($r_s = 0.436$, $r_s = 0.345$ and $r_s = 0.175$ respectively).

There was a significant positive correlation between years of exposure to silver fumes and P/C ratio ($r_s = 0.152$) which might be due to the critical effects of silver. Tubular proteinuria was generally accepted as the critical effect of long term and low level exposure to cadmium as seen in an industrial environment (Gonick, 2008). Madden and Fowler (2000) opined that the renal pathological effects of metals vary with the chemical form of the metal, the dose and acute or chronic nature of exposure.

PHASE II

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

It has been estimated that nearly half of all enzymes are metalloproteins (Waldron *et al.*, 2009) and vast numbers of metalloproteins remain uncharacterized (Cvetkovic *et al.*, 2010). The affinity of proteins to metals influenced by universal order of preference for biologically important divalent metals includes $Mg < Ca < Mn < Fe < Co < Ni < Cu < Zn$, which ranks the relative stability of complexes formed with each metal ion (Frausto da Silva and Williams, 2001). Several nonessential toxic metals

include mercury, cadmium and silver which form tight complexes with thiolates and obey an order of preference namely $Zn < Co < Pb < Cd < Cu/Ag < Hg$ (Palacios *et al.*, 2011a).

Metallothioneins (MTs) represent a universal family of polymorphic, ubiquitous and small cysteine rich metal binding polypeptides in mammals which are characterized into four extremely similar isoforms, designated MT1, MT2, MT3 and MT4 (Artells *et al.*, 2013). They are not considered to be vital for life, even though they are involved in many pathways which include sequestration of toxic (Cd and Hg) or essential (Zn and Cu) metals, scavenging of free radicals, infection and inflammation (Coyle *et al.*, 2002).

MT isoforms bind to multiple metals in two well defined metal thiolate clusters (Ngu and Stillman, 2009). A high degree of metal selectivity depends on the inherent properties of the MTs (Foster and Robinson, 2011).

Under physiological conditions, cellular zinc and copper ions bind to MTs through cysteine residues. But these metals can be displaced due to exposure to other heavy metals such as mercury, cadmium, silver, platinum and lead (Faller, 2010; Lindeque *et al.*, 2010). High metal binding ability of MTs confers vital role in metal cellular homeostasis, heavy metal detoxification and protect cells and tissues from heavy metals toxicity (Maret, 2009). Induction of MTs that symbolize metal detoxification in the body includes metal homeostasis to maintain the levels of trace elements and protect cells from damage by metal burden (He and Ma, 2009).

Metals bound MTs are considerably 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 (Bonneris *et al.*, 2005). There are several studies carried out in peripheral blood lymphocytes (PBLs) to know the expression of MTs *in vivo* and it was found that MTs are expressed in lymphocytes at relatively low level. But they can be induced appreciably with metal exposure (Ooi *et al.*, 2010; Lu *et al.*, 2001; Harley *et al.*, 1989; Enger *et al.*, 1983; Hildebrand *et al.*, 1979). Since PBLs can easily be collected, it is

practically possible to use them to determine MTs expression to reflect metal exposure (Lu *et al.*, 2001).

Hence, the present study was aimed to isolate, determine the molecular mass, quantify and identify the functional groups of the MTs from PBLs using gel filtration, SDS PAGE, HPLC and FTIR methods respectively.

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

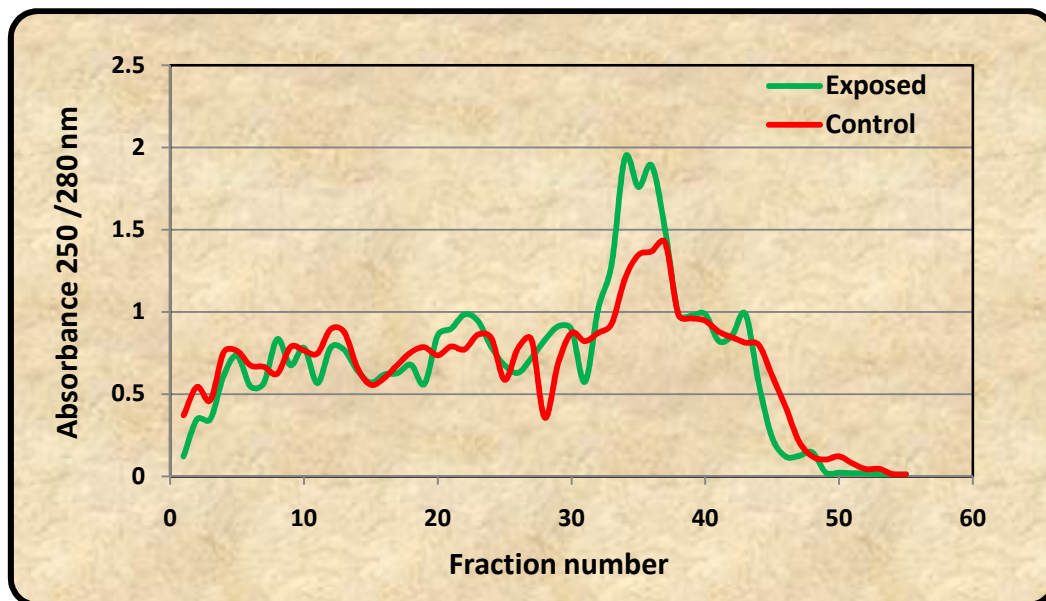
Metallothioneins (MTs) are authenticated biomarkers for metal exposure. They are differentiated by an extraordinarily high content of metal ions and form characteristic metal thiolate clusters through sulfhydryl or thiol (-SH) groups of cysteine (Dabrio *et al.*, 2002).

Workers who had high levels of metallothionein and silver content in serum were selected for the study. Peripheral blood lymphocytes (PBLs) were isolated from selected exposed (n=3) and control (n=3) group workers. Supernatant of the PBLs lysate was eluted in sephadex G-75 gel filtration column at the rate of 15 ml/hour and volume of each fraction was set as 1.5 ml. The absorbance of the eluted fractions was measured at 250 nm and 280 nm. As expected, the low molecular weight protein showed a high absorbance at 250 nm, a feature indicating the presence of mercaptide bonds with metals and low absorbance at 280nm confirmed absence of aromatic amino acids as it had already been shown for MT from various species (Wilhelmsen *et al.*, 2002).

According to Gulati *et al.* (1987), the ratio of absorbance at 250 nm / 280 nm more than 1 indicated the presence of mercaptide bonds. Hence absorbance ratio (A₂₅₀/ A₂₈₀) of each fraction was calculated in the study. The absorbance ratio of each fraction of controls and jewellery unit workers is shown in Figure 15.

Figure 15

Profiles of Sephadex G-75 Column eluted fractions from Peripheral Blood Lymphocytes of Selected Jewellery Unit Workers showing the absorbance 250/280 nm of the Sample Fractions



In the present study, 55 fractions were collected from both exposed and control groups with each fraction volume of 1.5 ml in which fractions 32, 33, 34, 35 and 36 in exposed group and fractions 34, 35, 36 and 37 in control group recorded an absorbance at 250 /280 nm more than 1 indicating the presence of mercaptide bonds. According to Palacios *et al.* (2011b), any protein or polypeptide characterized by a high content of cysteine residues with a low molecular weight give them a raised metal binding ability, thus proving the presence of metallothioneins (MTs). All the cysteine residues of MTs occur in the reduce form and are conjugated through mercaptide bonds with metal ions (Velisek, 2013). Hence the eluted fractions having absorbance ratio more than 1 in the present study could be considered as MTs fractions.

The present study results could be supported by the work of Fatur *et al.* (2002). They demonstrated that Superdex 75TM gel filtration profiles of supernatant from HepG2 cells exposed to 1 μ M CdCl₂ showed the position of MTs in the eluted fractions between 18 and 21.

Cytosol of mouse fibroblast cell lines incubated with CdCl₂ in the presences of thiols for 1 hour and separated through Sephadex G-75 column showed the presence of MTs in the flow through fractions between 28 and 33 (Suhy *et al.*, 1999).

Simes *et al.* (2003) reported that MTs separated and purified using gel permeation and ion exchange chromatography from cadmium exposed clams (*Ruditapes decussatus*) with a high A254/A280 absorbance ratio resolved four cadmium metallothioneins (Cd-MTs) and an absorption spectra of all four Cd-MTs peaks showed the presence of characteristic cadmium thiol (Cd –S) aggregates.

According to Prasad *et al.* (1998), Indian childhood cirrhosis liver cytosol subjected to Sephadex G-75 gel filtration column to determine the heat stable copper binding proteins showed one major peak representing the position of MT in the flow through fractions between 48 and 64. Lysate of cadmium (Cd²⁺) exposed human peripheral blood lymphocytes loaded on a Sephadex G-75 column showed a low molecular weight peak representing the position of MTs in the eluted fractions between 31 and 42 (Sone *et al.*, 1988).

From the study, fractions with 250/280 nm absorbance ratio more than 1 were pooled together, concentrated and assayed for sulfhydryl content of isolated metallothioneins.

4.2.2 Sulfhydryl content of isolated metallothioneins from the chromatographic fractions

Figure 16 features the level of sulfhydryl content of isolated metallothioneins in the control and exposed group.

In the present study total sulfhydryl content of PBLs was found to be raised significantly ($p < 0.05$) in exposed group compared to control group which implied that elevated level of MTs might be induced in response to metal exposure and it might be actively involved in protection against oxidative stress.

Thiols can be described as a group of compounds rich in sulfhydryl (–SH) moieties. This moiety is extremely reactive and is found to attach with both organic and inorganic molecules. The most important characteristic of this moiety is its tightly binding ability to metal ions (Hynek *et al.*, 2012).

The thiol or mercaptan or sulfhydryl (–SH) side chain of cysteine is prone to react with reactive oxygen species (ROS) which gives rise to an oxidative post translational modification that can functionally regulate the protein (Rudyk and Eaton, 2014).

Several studies have shown that thiols play vital roles in a number of biologically imperative processes including scavenging of ROS and regulating the levels of biologically important metal ions (Adam *et al.*, 2007). Metallothioneins are considered as proteins with unusual content of cysteine moieties participating in the detoxification of heavy metals, since they have the capability to bind heavy metal ions via sulfhydryl groups of cysteine units and also they maintain oxidation and reduction status of the cell (Shariati *et al.*, 2011).

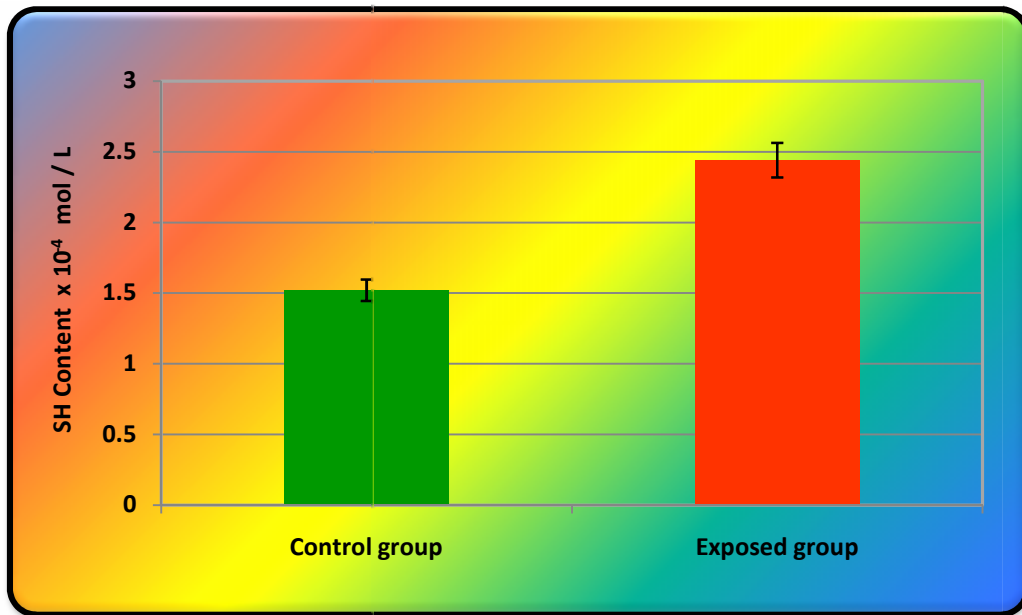
Compounds rich in free sulfhydryl (–SH) moieties interact with heavy metal ions after entering through cell transporters and these thiol groups are a part of peptides or proteins directly connected with the defensive mechanisms in a cell against adverse effects of metal ions (Zitka *et al.*, 2010).

MTs and glutathione (GSH/GSSG) contain sulphhydryl (thiol) groups which bind to a variety of metals (Kovarova and Svobodova, 2009). Total content of thiols and MTs determined in larvae sampled from eight fields polluted with various levels of heavy metals were higher in comparison to control ones as reported by Fabrik *et al.* (2008). Goulart *et al.* (2005) opined that thiol antioxidant (GSH) was significantly higher in PBLs in the welders group when compared with control group and suggested that substantially high molar concentration of GSH in lymphocytes are more readily available to react with absorbed metals.

The thiol content was increased in marine diatom *Phaeodactylum tricornutum* cells exposed to cadmium (20 mg/l) compared to total thiol group of unexposed cells and also total thiol content did not change in unexposed cells during the experiment. Highest total thiol content in the cadmium exposed cells was obtained after 10 days as 5.61 ± 0.23 nmol (Torres *et al.*, 1997).

Figure 16

Sulphydryl (-SH) Contents of isolated MTs from PBLs in Controls and Jewellery Unit Workers



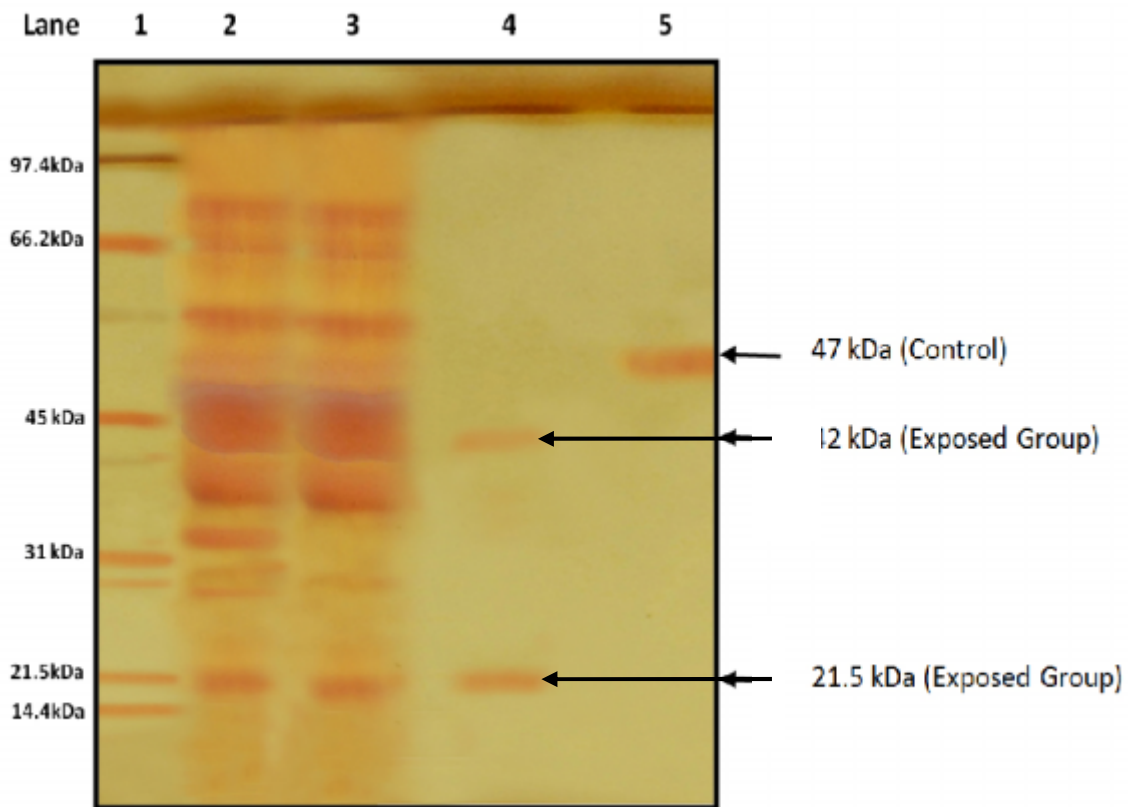
4.2.3 Molecular mass of isolated metallothioneins from PBLs of jewellery unit workers

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS - PAGE) has been used as a valuable and qualitative technique to identify the molecular mass of the specific protein in the samples (Kovenden *et al.*, 2013). SDS-PAGE electrophoresis with silver staining is probably a sensitive indicator of the early effects of metals on protein.

Molecular weight of isolated metallothioneins was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE protein profile of peripheral blood lymphocytes of exposed workers and controls are presented in Plate 2.

Plate 2

SDS-PAGE Protein Profile of Peripheral Blood Lymphocytes of Exposed Workers and Controls



Lane 1 shows the protein standard marker

2 and 3 show the protein profile of PBLs of exposed and control group respectively

4 shows the gel filtrated protein profile of PBLs of exposed group

5 shows the gel filtrated protein profile of PBLs of control group

The arrow marks indicate the gel filtrated protein bands of 21.5, 42 kDa of exposed workers and 47 kDa of controls

In the present study, protein markers (14.4- 97.5 KDa) were used as reference standard in order to identify molecular mass of protein from the collected fractions of PBLs of exposed and control group (as shown in lane 1 of Plate 2 electropherogram). Gel was read by gel documentation system (Bio-Rad).

Even though numerous bands were monitored in the protein profile of PBLs of exposed and control group on the stained gel, only 2 bands and one band were observed in the gel filtrated protein fractions of exposed and control group respectively.

Molecular mass of the gel filtrated proteins of PBLs was determined in the study as 21.5kDa and 42 kDa for exposed group demonstrated in lane 4 and 47kDa for control group illustrated in lane 5 of Plate 2 electropherogram.

In the present study low molecular weight protein band (21.5 kDa) observed on the gel was considered as MTs with numerous sulfhydryl group (as shown in Figure 16). MTs are low molecular weight proteins (6 – 10 kDa). However, in the present study high molecular mass (21.5 kDa) obtained might be due to the formation of multimeric complex of multiple sulfhydryl groups of MT.

A study on the identification of MT in *Pleurodeles waltii* showed that the protein displayed its molecular weight as 28 kDa. This result does not fit well with the expected low molecular weight (6 kDa) of MT. This discrepancy can be explained taking into account the unusual properties of MT. The multiple sulfhydryl groups of MT lead to the formation of multimeric complexes and then to form as high molecular weight protein (Mounaji *et al.*, 2002).

Vergani *et al.* (2005) compared two MTs from *Mytilus galloprovincialis* (MT 10) and *Oncorhynchus mykiss* (MT A). Both MTs showed metal binding ability with cadmium. Both proteins were analyzed by gel electrophoresis and their result suggested that dark band at high molecular mass (12 – 18 kDa) might be due to oxidative polymerization of the MT molecules.

Some high molecular weight proteins (42 kDa in lane 4 of exposed group and 47 kDa in lane 5 of control) observed in the present study might be due to inefficient heat denaturation procedures which is supported by the findings of Simes *et al.* (2003).

According to Zhukovskaya *et al.* (2012), *Mizuhopecten yessoensis* can synthesize high molecular weight proteins in the presence of cadmium which have a number of properties similar to MT with the molecular mass of 72 kDa and 43 kDa.

Interestingly, low molecular weight protein band did not appear in the gel filtrated control sample which might be due the abnormalities or irregularities of MTs by forming adduct with some other protein present in the gel filtrated protein sample. This complex

showed a high molecular weight dark band (47 kDa) as shown in the lane 5 of Plate 2 that could be supported by the observation of Qureshi *et al.* (2013).

In SDS-PAGE, low molecular weight protein band was not present in the gel filtrated control sample but experimental group sample showed low molecular weight protein band which might be due to the exposure to silver stimulated synthesis of MT. The above result might reveal that MT might be involved in detoxification of metal toxicity. Induction of MT has been considered as one of the defense mechanisms that protects the cell from the reactive transition of metal toxicity (Cherian, 1995).

Sone *et al.* (1988) separated a family of Cd²⁺ binding proteins from Cd²⁺ exposed human peripheral blood lymphocytes (PBL) by gel filtration and characterized them by SDS PAGE. They revealed that electrophoretic mobility of the isolated proteins was closely related to MTs of HeLa cells derived from human cervical carcinoma. These proteins were rich in cysteine residues and they were detected only in Cd²⁺ exposed lymphocytes and did not appear in unexposed lymphocytes.

Hauser-Davis *et al.* (2012) conducted 1D/2D gel electrophoresis to verify the presence of MT in fish bile and showed the presence of MT which could be induced by heavy metals and the data revealed that bile MTs pursue similar trend as in liver.

Electrophoretic (15% SDS Polyacrylamide gel) analysis of cytoplasmic proteins in human peripheral blood lymphocytes exposed to heavy metals namely CdCl₂, HgCl₂ or ZnSO₄ illustrated the presence of carboxymethylated thioneins on the gel (Yamada and Koizumi, 1991).

Electropherogram of cytosolic extract of liver and kidney of freshwater fish (*Cyprinus carpio*) exposed to arsenic trioxide (As₂O₃) at different concentrations (5, 15 and 20 ppm) showed the presence of MT with molecular weight of 5 Kda and 6 Kda respectively. But low molecular weight protein band was not observed on the gel in the control as reported by Kovendan *et al.* (2013).

The characteristic denaturing SDS-PAGE electropherograms of liver and kidney homogenate from fish, human and pig blood serum samples were discussed by Krizkova *et al.* (2009). They were able to detect MT in all the samples with molecular mass of less than 10 kDa.

4.2.4 Quantification of isolated metallothioneins by HPLC

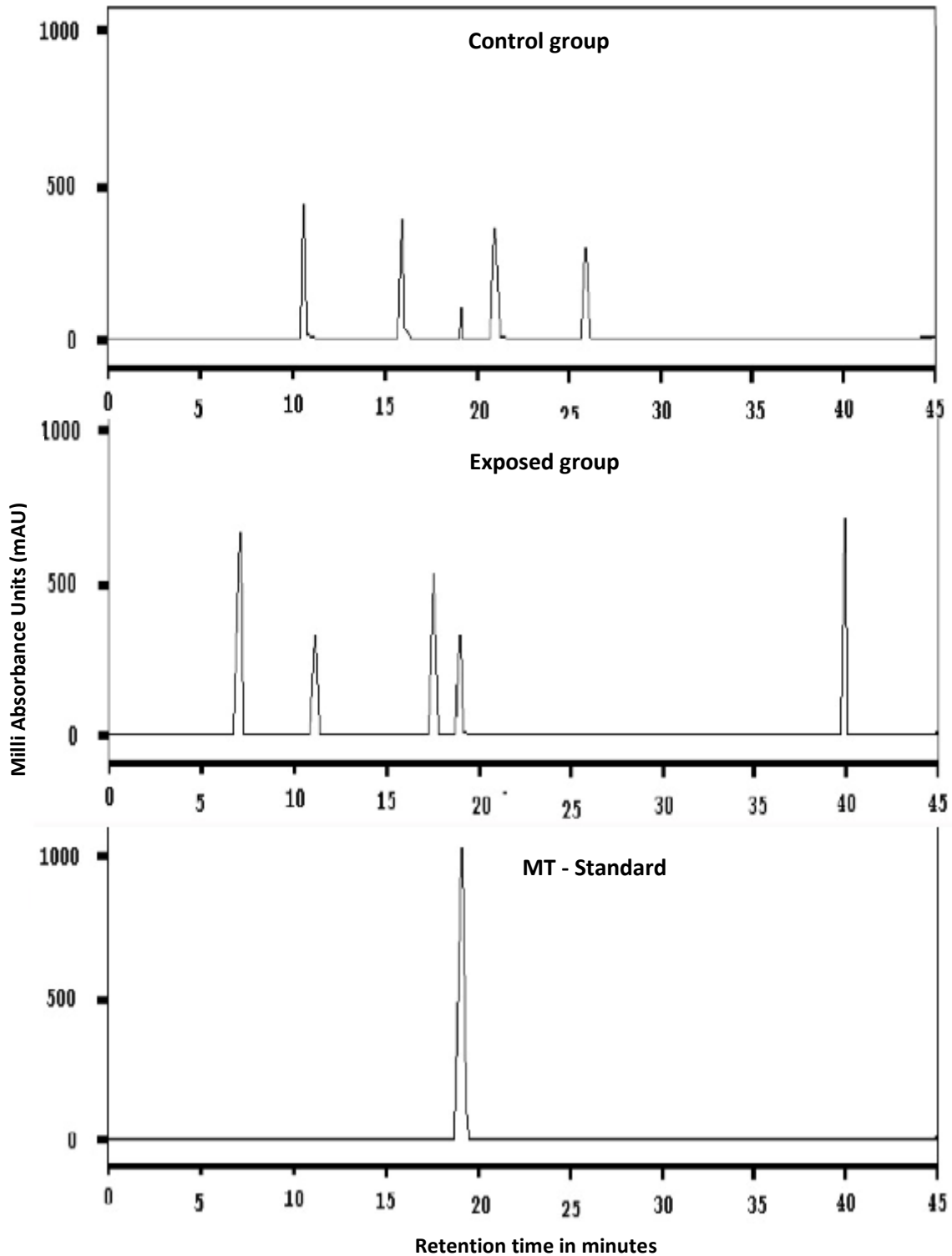
HPLC is a sensitive and convenient determination method for MTs used to clarify their physiological roles, including Zn - MT, Cd - MT and Cu - MT from cultured cells as well as in animal tissues using commercially available rabbit MT as a standard (Miyairi *et al.*, 1998).

Figure 17 gives the details of high performance liquid chromatography (HPLC) spectra of metallothionein in PBLs of exposed workers and controls.

In the present study, the retention time for control, exposed group and horse kidney metallothionein standard (Sigma) were 18.97, 19.12 and 19.02 minutes respectively as analyzed by HPLC. MT was estimated as 40.3 % and 11.27 % present in experimental and control samples respectively. This result revealed an elevation of metallothionein in exposed group compared to control group and provided evidence that MTs could be induced by metal exposure.

Figure 17

HPLC Spectra of Metallothionein in PBLs of Exposed Workers and Controls



Earlier works on MTs supporting the presence study results are presented in Table 17.

Table 17
Earlier studies on Metallothioneins from Various Species

Earlier work	Findings	Researchers	Year
MT-I and MT-II were separated and quantified by anion exchange HPLC coupled with atomic absorption spectrophotometry (AAS) from rat liver	Both the MT isoforms were detected in cadmium and zinc treated rats with the elution at 7.5 and 10.4 minutes, respectively	Lehman and Klaassen	1986
MTs (MT-I and MT-II) were separated from mouse and rabbit livers by HPLC anion exchange column and the peak holding MTs was determined by atomic absorption spectrophotometry.	MT-I and MT-II were eluted at 15.9 and 19.3 minutes respectively	Pan <i>et al.</i>	1991
MT in rat liver was separated in Superdex 75 high performance gel filtration column and determined by HPLC	MT was detected by a UV spectrophotometric detector at 250nm and showed retention time at 14.3 minute	Jin <i>et al.</i>	1993
MTs were extracted from the liver cytosol of humans using size exclusion and anion exchange chromatography	MT content in the samples was calculated by generally accepted metal/protein ratios for Cu (12:1), Cd (7:1) and Zn (7:1) in which highest levels of Cu-MT was found in primary biliary cirrhosis	Wolf <i>et al.</i>	2000

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Table 17 (Contd...)

Earlier work	Findings	Researchers	Year
<p>Size exclusion coupling of high performance liquid chromatography with inductively coupled plasma - time of flight mass spectrometry (SE-HPLC-ICP-TOFMS) profiles of hepatic and renal cytosols of carp (<i>Cyprinus carpio</i>) exposed to different concentrations of cadmium (0.1, 1.0, 2.5 and 10 mM Cd) at 96 hours was studied</p>	<p>Cd-MT I and II were obtained with the retention times similar to standards of rabbit liver MT I and MT II.</p>	<p>Campehouth <i>et al.</i></p>	<p>2004</p>
<p>Zinc bound MT and other cytosolic protein fractions with distinct molecular weight in human peripheral blood mononuclear cells (PBMC) were determined by a simple ultra high resolution size exclusion HPLC (UHR-SEC-HPLC) system with online detection by ICP-MS</p>	<p>MT was quantified in very small quantity of sample.</p>	<p>Malavolta <i>et al.</i></p>	<p>2007</p>
<p>MT was quantified in sea bass (<i>Dicentrarchus labrax</i>) intraperitoneally injected with different concentration of copper, cadmium and mercury (50-250 mg/ kg wet weight) after 48 hour exposure by reversed phase high performance liquid chromatography coupled to fluorescence detection (RP-HPLC-FD)</p>	<p>Metals could induce MT in the liver of sea bass with retention time at 16.8 minute</p>	<p>Jebali <i>et al.</i></p>	<p>2008</p>

Table 17 (Contd...)

Earlier work	Findings	Researchers	Year
MT was studied by reversed phase high performance liquid chromatography coupled to fluorescence detection (RP-HPLC-FD) from the digestive gland of various clam species and liver of fish injected with different metal doses (Cu, Cd and Hg).	MT peak was resolved	Alhama <i>et al.</i>	2011
Three methods [1D/2D gel electrophoresis, spectrophotometric analyses and step size exclusion column HPLC with inductively coupled plasma mass spectrometry (SEC-HPLC-ICPMS)] were carried out to verify the presence of MTs in fish bile.	All the three methods showed the existence of MT	Hauser-Davis <i>et al.</i>	2012
Soluble extracts of liver and brain from 129/Sv mice either treated with cadmium or untreated were analyzed by one step size exclusion column HPLC with inductively coupled plasma mass spectrometry (SEC HPLC-ICP-MS).	MT-1, MT-2 and MT-3 were separated from cadmium treated mice with the retention times of 8.6, 8.1 and 7.6 minutes respectively	Kameo <i>et al.</i>	2014

4.2.5 FTIR spectral analysis of isolated metallothioneins

Fourier transformed infrared (FTIR) spectroscopy determines the absorption of infrared radiation by chemical bonds in functional groups of molecules. The frequency range of absorption by these molecules is correlated with the structure of the molecules

(Eysel et al., 1993). FTIR spectra were illustrated in the present study to monitor the changes in the functional groups in isolated MTs induced by metal ions. The IR spectra were measured between wavenumber 4000 and 400 cm^{-1} . The FTIR spectrum for MTs of exposed group workers was compared with control FTIR spectrum to analyze the presence of stress factors produced during metal exposure.

Figures 18 and 19 present the details of FTIR spectra of MTs from PBLs of control and exposed group workers.

FTIR spectra of jewellery unit worker and control showed broad bands at 3331.21 cm^{-1} and 3288.84 cm^{-1} for exposed workers and 3337.95 cm^{-1} and 3331.21 cm^{-1} for control. This result was in accordance with the report of Wolkers *et al.* (1998) who reported the bands around 3330 - 3370 cm^{-1} representing O-H and N-H stretching vibrations that are mainly generated by proteins.

The oxidation of cysteine (R-SH) can result in the liberation of bound metal and the formation of cystine (R-S-S-R). Cysteine presents in three main states as coordinate with metals or formation of disulfide bonds or free (Maret, 2003). Free cysteine in MTs can be evidenced by -SH stretching band that appears in 2600-2500 cm^{-1} wavenumber. This band is a proof for the presence of thiol (-SH) or sulfhydryl moiety in MTs and no other groups give rise to bands in this region as reported by Nemeth (2007).

Since this band has not been found in MTs of both the exposed worker and control in the study, it can be concluded that these MTs do not contain free cysteine residues.

The differences in vibration of amide bonds are due to differential hydrogen bonding among aminoacid residues. The differential patterns in hydrogen bonding along with geometric orientations of amide bonds in α helices, β sheet and random coil structures permit the diverse vibration frequencies associated with the individual secondary structure folding. Vibration involves in C=O, C-N and N-H groups of amide bond which results in distinguishing spectral features of protein (Stehfest *et al.*, 2005). Three major spectral regions designated 1700 -1600 cm^{-1} of amide I, 1600 -1500 cm^{-1}

of amide II and 1320 – 1205 cm^{-1} of amide III have been identified by Ozaki *et al.* (2003) based on theoretical and experimental studies.

Figure 18
FTIR Spectrum of MTs from PBLs of Control

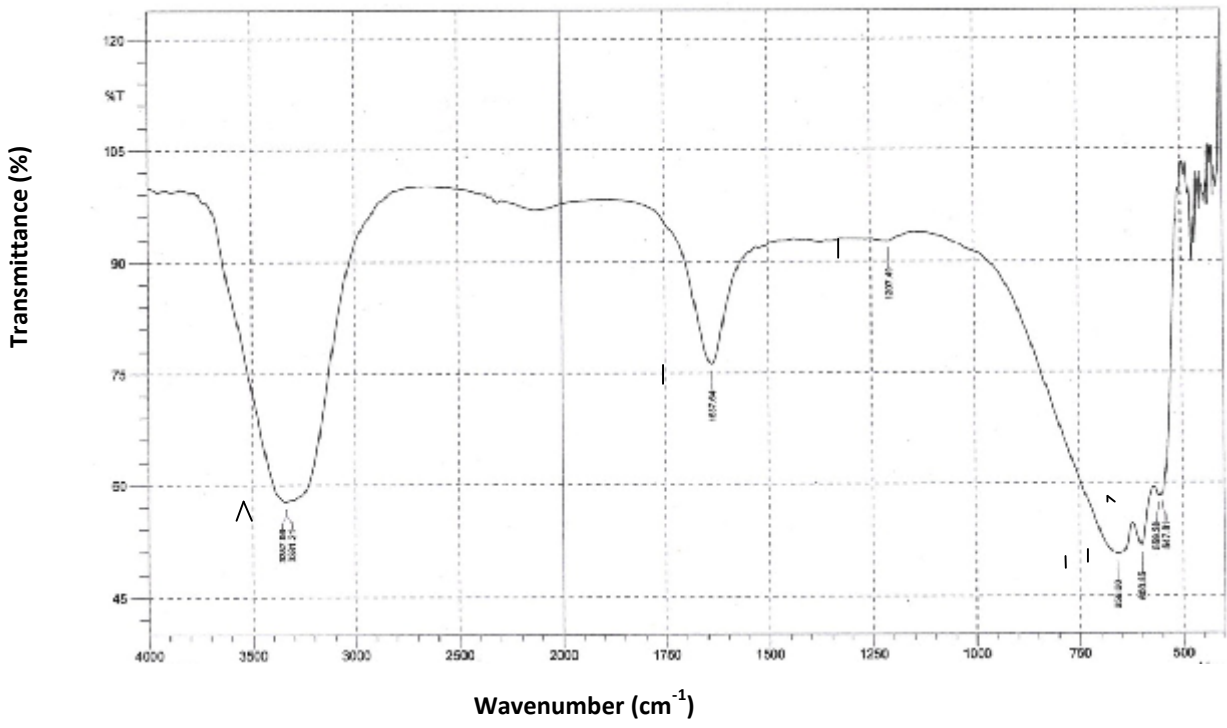
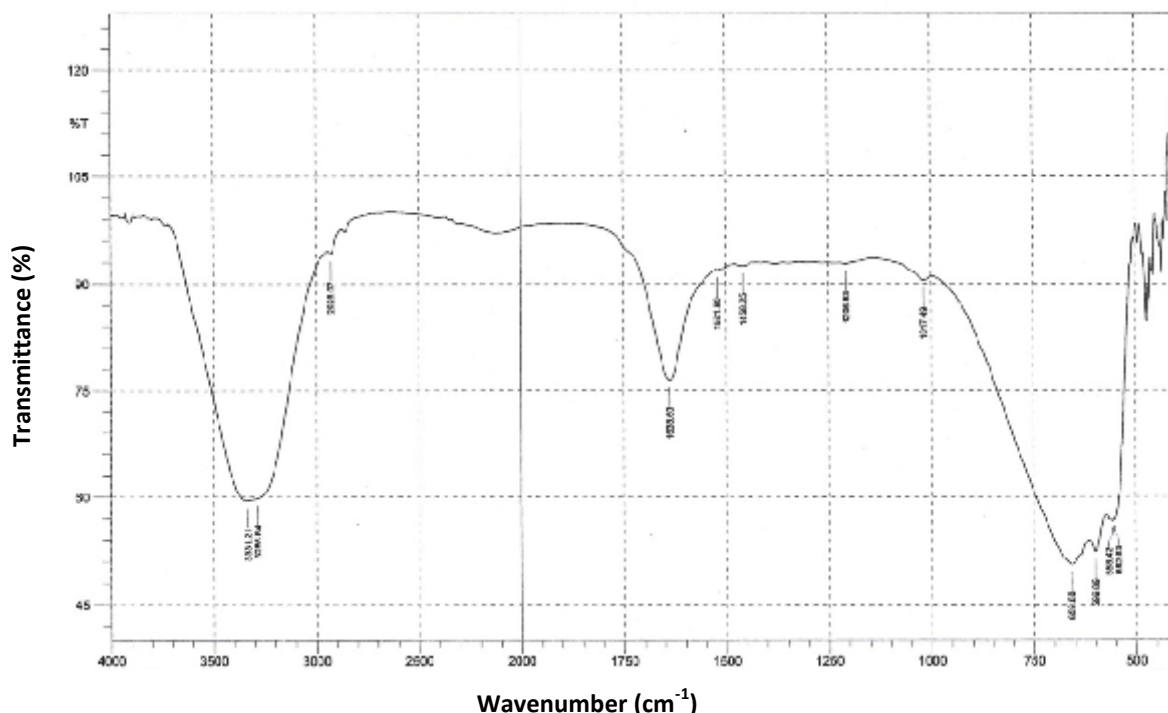


Figure 19
FTIR Spectrum of MTs from PBLs of Jewellery Unit Workers



The absorption bands of the proteins generally found between 1800 and 1500 cm^{-1} consisted of amide-I and amide - II bands as reported by Surewicz *et al.* (1993) and Stehfest *et al.* (2005). Amide - I and II bands are particularly useful to determine the changes in the IR absorption of proteins under metal stress. In the present study, bands at 1637.64 cm^{-1} and 1638.60 cm^{-1} were observed in the FTIR spectra of control and exposed worker respectively, indicating the presence of amide - I group (C=O stretch). Amide - I region (1700-1600 cm^{-1}) mainly corresponds to C=O stretching vibrations of polypeptide which can help in the identification of the overall protein conformation and content as observed by Nishina *et al.* (2007).

According to Wei *et al.* (2009), bands at 1656 cm^{-1} and 1621 cm^{-1} represent the alpha turn and beta sheet of secondary structure of protein. In the present study, there were no such bands in MT samples of both experimental worker and control which did not indicate any secondary structure (α turn and β sheet) of protein.

The conformational dynamics and structural properties of Antarctic fish (*Notothenia coriiceps*) and mouse MTs were studied by FTIR and fluorescence

spectroscopy by Capasso *et al.* (2002). FTIR data revealed that fish and mouse MTs lack secondary structural elements such as alpha helices and beta sheets.

In the present study, band at 1521.90 cm^{-1} in MT in experimental group worker indicated the presence of amide - II band. But there was no such band in control participant. Changes in amide – II region of exposed group worker compared to control might be due to metal stress. Wei *et al.* (2009) reported that cadmium stressed changes were monitored in the amide II region positioned at around $1544 - 1500\text{ cm}^{-1}$ of proteins. According to Othman, (2009) amide II absorption is due to N-H bending vibration coupled to C-N stretching and it is seen at $1560 - 1500\text{ cm}^{-1}$.

A band at 1458.25 cm^{-1} believed to be related to protein was observed in the exposed worker of the study which was not observed in the control. Similar to the present study observation, Lewis *et al.* (2010) reported that a broad region of peaks assumed to be associated with proteins was noticed between 1400 cm^{-1} and 1450 cm^{-1} .

In the present study, both the exposed worker and control showed the bands at 1207.40 cm^{-1} for control and 1206.53 cm^{-1} for experimental group indicating the presence of amide – III band in MT. Amide –III band although less intense than amide - I, can facilitate the structural analysis of protein and amide III absorption is obtained due to C-N stretching and N-H in plane at $1350 - 1205\text{ cm}^{-1}$ as suggested by Zhao (2008).

Band at 659.68 cm^{-1} was seen in the exposed worker and control of the study indicating the thioethers ($\text{CH}_3 - \text{S}$) stretch in MT. Bands at 599.89 cm^{-1} of exposed worker and 600.85 cm^{-1} of control indicated the presence of disulfides (S-S) stretch. From the study bands at 559.38 cm^{-1} of control and 558.42 and 552.63 cm^{-1} of exposed worker specified the presence of disulfides (C-S-S-C) stretch. This result showed that more number of disulfide bonds present in MT of exposed worker compared to control revealed that metals actively bind to sulfur atoms of MTs through mercaptide bonds. However, it could be suggested that metal free MTs take a random coil structure consisting of disulfide bonds that is created for structural stability of proteins.

According to Coates (2000), bands at $660 - 630 \text{ cm}^{-1}$, $620 - 600 \text{ cm}^{-1}$ and $705 - 550 \text{ cm}^{-1}$ showed the presence of thioethers ($\text{CH}_3 - \text{S}$) stretch, disulfides (S-S) stretch and disulfides (C-S-S-C) stretch, respectively. MTs have strong metal binding properties and metals bind to MTs through the sulfur atoms in cysteines. This is done through two domains of the MT designated as alpha domain and beta domain as reported by Klaassen *et al.* (1999).

MTs do not contain disulfide bonds in the primary structure and do not form alpha helices or beta sheets as secondary structures. Hence metal free MTs take a random coil as three dimensional structures to retain their stability (Stillman, 1995). FTIR spectra of MTs of the study might indicate that they do not contain secondary structure (α helix and β sheet) and also consist of disulfide bonds in exposed worker and control samples proving that the isolated MTs are more flexible and take random coil structure.

PHASE III

4.3 Expression of Metallothioneins in the Cultured Lymphocytes of the Selected Workers on Exposure to Silver ions

Metallothioneins (MTs) are carrier proteins that play an important role in detoxification processes of metals in humans. MT genes are ubiquitously expressed in many tissues in basal levels and their expressions are induced readily by many factors, specifically by metal ions. Reports of laboratory and field investigations had revealed that MTs are induced during chronic and acute exposure to metals. Several studies on MTs have proved that they are involved in the absorption, transport and excretion of heavy metals. MTs provide a protective role against toxic effects of metals by sequestering and thus reducing the amount of free metal ions. Thus, MTs expression could be used as specific biomarker for metal exposure in industrial health. Once MTs are saturated with metals excess metal ions spill over into other cellular compartments and cause pathological lesions (Ganguly *et al.*, 1996; Ooi *et al.*, 2010).

Human peripheral blood lymphocytes (PBLs) generate MTs in response to a variety of heavy metals. Therefore, MTs from PBLs could be used as a candidate

marker that represents the biological effect of heavy metals (Yamada and Koizumi, 2001).

To evaluate this, experiments were designed in such a way that PBLs were exposed to various concentrations of silver nitrate (AgNO_3) through cell culture method. Viability of different concentrations of AgNO_3 exposed cells was estimated using 0.4% trypan blue after 24 hours to find out the number of viable cells in the cell suspension. Also the present study was planned to determine the mitochondrial activity of living cells of cultured lymphocytes treated with different concentrations of AgNO_3 at different time intervals by MTT assay. An attempt was made to analyze the internal changes happening in AgNO_3 exposed lymphocytes ($4 \mu\text{M}$) using Transmission Electron Microscope (TEM) after 24 hours and compared with unexposed (control) lymphocytes. The present study was also aimed to isolate, quantify and identify the functional groups of the MTs from cultured PBLs using gel filtration, HPLC and FTIR methods respectively. The present study was attempted to find out the free radical scavenging activity of isolated metallothioneins from AgNO_3 treated PBLs and compared with control.

4.3.1 Cell viability of silver nitrate (AgNO_3) exposed peripheral blood lymphocytes

Table 18 presents percent viability of lymphocytes treated with different concentrations of AgNO_3 after 24 hours.

Table 18
Per cent Viability of Lymphocytes treated with Different Concentrations of AgNO_3 after 24 Hours

Concentration of AgNO_3 (μM)	Percent Viability (%)
Control	95
0.5	93
1	92
2	80
4	67

When PBLs were exposed to 0.5, 1, 2 and 4 μM AgNO_3 for 24 hours, a concentration dependent decrease in the number of viable cells was observed at AgNO_3 concentration higher than 1 μM . Concentration of AgNO_3 up to 1 μM did not dramatically lower the cell viability (93% in 0.5 μM and 92% in 1 μM AgNO_3 treated cells) when compared to control (95%) after 24 hours. But concentrations of AgNO_3 namely 2 and 4 μM were shown to noticeably decrease the viable cells (80% and 67% respectively) after 24 hours when compared to control. The extent of cell death could be accounted for the oxidative stress due to AgNO_3 exposure.

Worth *et al.* (2001) studied the effect of mercury in neutrophils at different concentrations namely 2.5 μM , 5 μM , 7.5 μM and 10 μM and compared with control cells. They also revealed that concentrations up to 10 μM did not dramatically lower cell viability when compared to control ($80 \pm 6\%$ and $91 \pm 6\%$ respectively). As the dose of mercury was increased to 10 μM , the percentage of polarized neutrophils was decreased in a dose dependent manner from $53 \pm 4\%$ to $21 \pm 2\%$.

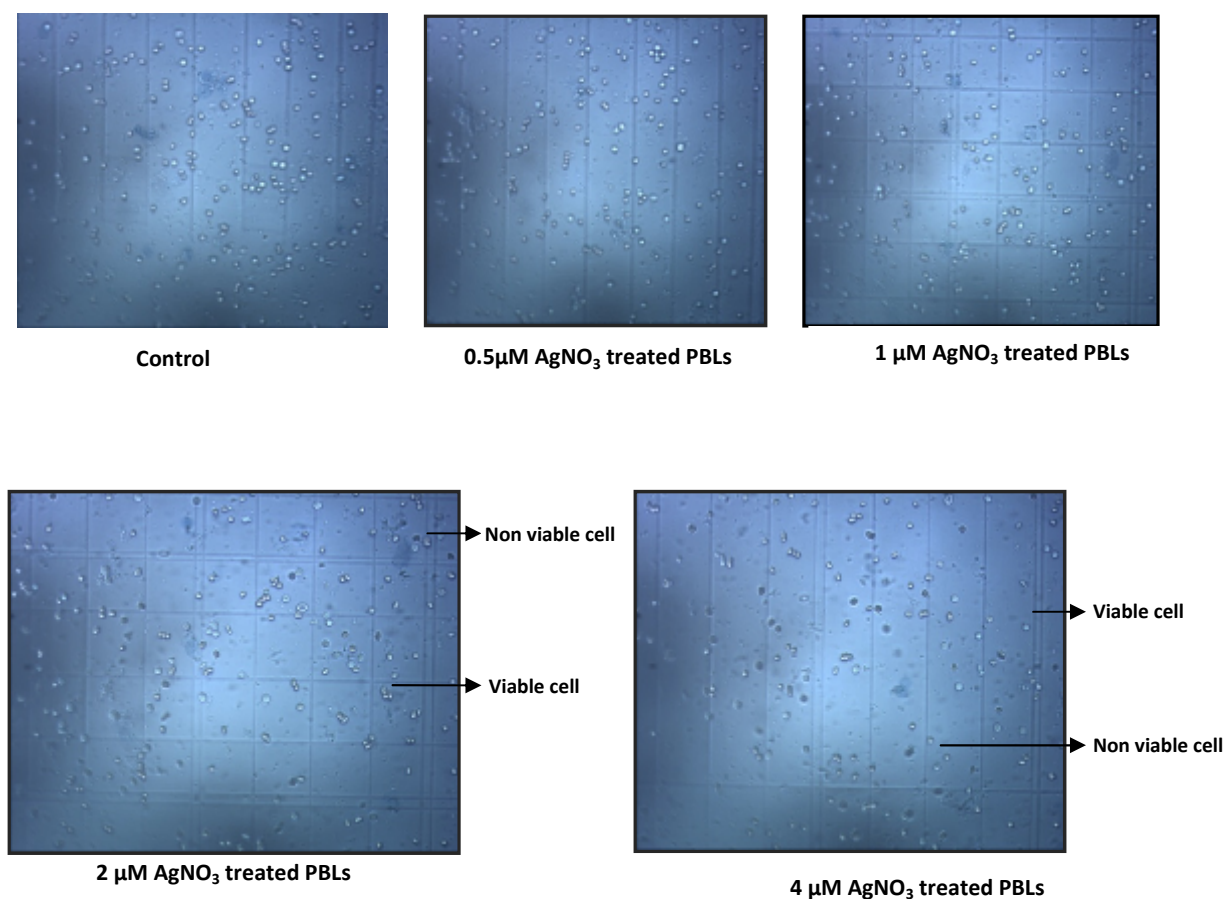
Parran *et al.* (2001) studied the effects of mercury compounds on cell survival in which unprimed PC12 cells were exposed to various concentrations of either CH_3Hg or HgCl_2 for 24 hours and cell viability was examined using trypan blue exclusion. Their findings showed that HgCl_2 and CH_3Hg exposure produced a concentration dependent decrease in cell viability.

Three different polymerized and unpolymerized resins based tooth coloured materials namely nano, flowable and compomer materials were evaluated to study the cytotoxicity on human lymphocytes by trypan blue exclusion method at different time intervals of 24, 48 and 72 hours. Among three different materials, mean percentage viability of nano and flowable materials were found to be maximum with the mean values of $69.07 \pm 11.85\%$ and $59.24 \pm 13.60\%$ respectively. The mean percentage viability of negative control was found to be $89.39 \pm 2.17\%$ as reported by Srivastava *et al.* (2010). They also revealed that all the resins were found to be cytotoxic to human lymphocyte and cytotoxicity was recorded highest after 24 hours.

In the present study, viability of different concentrations of AgNO_3 treated cultured lymphocytes after 24 hours using 0.4% trypan blue are shown in Plate 3 in which unstained and stained cells with trypan blue dye were counted as viable and non viable respectively.

Plate 3

Viability of Different Concentrations of AgNO_3 Treated Cultured Lymphocytes after 24 Hours using 0.4% Trypan Blue



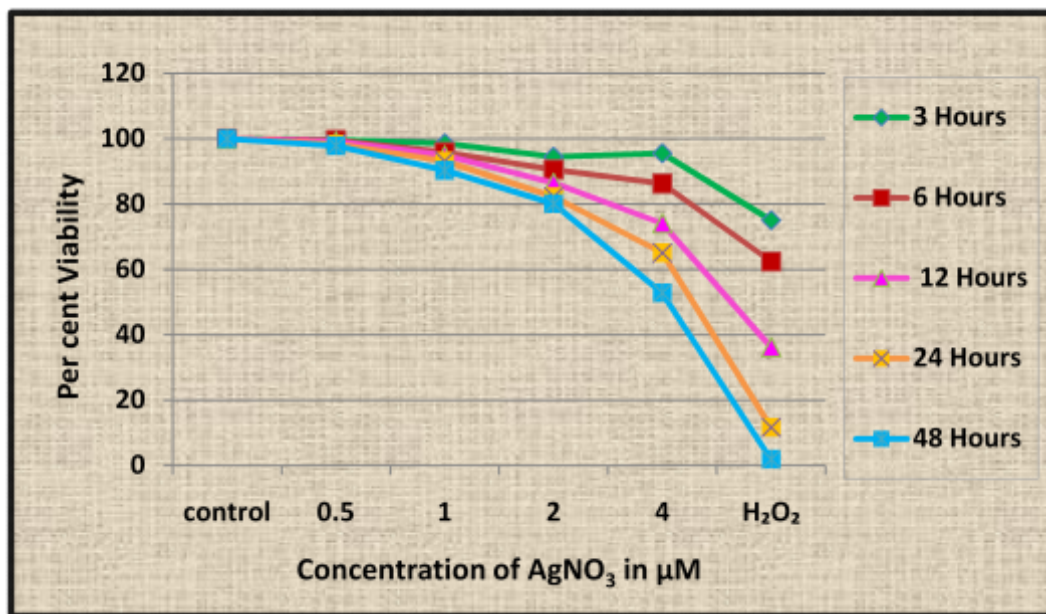
4.3.2 Cytotoxicity of AgNO_3 to human lymphocytes *in vitro*

The extent of cell survival on the exposure to different concentrations of AgNO_3 (0.5, 1, 2 and 4 μM) was studied by MTT assay. The per cent cell viability was quantified at different time intervals namely 3, 6, 12, 24 and 48 hours and are illustrated in Figure 20. The values of untreated or negative control were fixed as 100% and the relative per

cent viabilities in the other different concentrations of AgNO_3 exposed groups were calculated. The data are expressed as the mean of triplicates of culture PBLs.

Figure 20

Viability of Peripheral Blood Lymphocytes after Exposure to Different Concentrations of AgNO_3 at Different Time Intervals



At each time point lymphocytes were harvested and washed in fresh RPMI prior to analysis of cell viability by MTT assay. Exposure to 0.5, 1, 2 and 4 μM concentrations of AgNO_3 helped to assess cytotoxicity to PBLs from Ag^+ ions for a time period up to 48 hours. Lymphocytes were also exposed to a single dose of H_2O_2 (50 μM) for the same time points as an indicator of cytotoxicity. H_2O_2 is a chemical known to cause loss of cell viability by oxidative stress which was considered as positive control.

H_2O_2 caused loss of cell viability in a time dependent manner in which approximately 25%, 38%, 64%, 88% and 98% loss of cell viability were noted after 3, 6, 12, 24 and 48 hours respectively. AgNO_3 caused a dose and time dependent loss of cell viability in PBLs with approximately 52% of cells remaining viable at the highest dose (4 μM) after 48 hours. At low doses of AgNO_3 (0.5 and 1 μM), there was a little loss of cell viability observed at all time points.

The results of the present study indicated that AgNO₃ induced the synthesis of metalloproteins (metallothioneins) and protected the cells from oxidative stress at low doses (0.5 and 1 µM). However, at higher doses (2 µM and 4 µM) of AgNO₃ exposure, the observed cytotoxicity to PBLs might be due to the accumulation of AgNO₃ in the cells.

Several studies have validated MTT assay as an appropriate tool for quantifying the extent of cell survival in various types of cells. Kawata *et al.* (2009) demonstrated cell viability assay in which silver nanoparticles (Ag-NPs) accelerated cell proliferation at low doses (<0.5 mg/L), that was supported by the DNA microarray analysis proving significant induction of genes associated with cell cycle progression. However, at higher doses (>1.0 mg/L) of Ag-NPs exposure, a significant cytotoxicity was revealed which induced abnormal cellular morphology, gaining an irregular shape and displaying cellular shrinkage.

An *in vitro* cytotoxic effect of silver nanoparticles was monitored against HL60 cell line and viability of tumour cells (HL-60) was validated using MTT assay by Sulaiman *et al.* (2013). They noticed dose dependent loss of cell viability in the HL-60 cells by the exposure of silver nanoparticles. After six hours of treatment, the silver nanoparticles at concentration of 2 mM seemed to decrease the viability of HL-60 cells to 44 % from the initial level. They also reported that longer exposures resulted in additional toxicity to the cells and reached to 80% dead cells after 24 hour of incubation.

4.3.3 Analysis of uptake of AgNO₃ by cultured lymphocytes using Transmission Electron Microscope (TEM)

In the present study, TEM analysis of PBLs showed the accumulation of electron dense precipitates after 24 hours of incubation in presence of 4µM AgNO₃. At higher magnifications it was evident that the nanoparticle was precipitated on the cells. This precipitate was absent when AgNO₃ was not added to the RPMI 1640 medium.

The present study was supported by the report of Henry *et al.* (2013) who demonstrated that *L. interrogans* strain L521 and *L. interrogans* sv *Lai la4131* mutant L522 were cultured in EMJH medium supplemented with 360 µM FeSO₄ in which

transmission electron microscopy showed the accumulation of electron dense material inside the cells.

According to Wang *et al.* (2010), TEM analysis of thin sections of *S. oneidensis* MR-1 cells grown for 24 hours anaerobically in presence of 0 - 100 μM of Ag(I) showed the accumulation of electron dense precipitates of 10 nm diameter around the cell after 24 hours of incubation in the presence of 10 μM Ag(I). Precipitation was obtained most probably due to the reduction of Ag(I) to Ag(0) at the surface of the cells. At higher magnifications, it was apparent that the nanoparticles were precipitated on the outside of the outer membranes of the Gram negative bacterial cells. These precipitates were absent when Ag(I) was not added to the growth medium.

The present study reports also revealed that at higher concentration (4 μM), metal can accumulate within the cell proposing that metal is able to cross the membrane to enter inside the cell. The present study results are supported by the findings of Wang *et al.* (2010); Feng *et al.* (2000); Simpson, (2003). They showed that at higher concentration (10 μM) the metal was accumulated within the cell as nanoparticles of Ag(0), suggesting that the metal was able to enter the cell either as reduced nanoparticles formed on the cell surface or as Ag(I) ions that were subsequently reduced and precipitated within the cytoplasm.

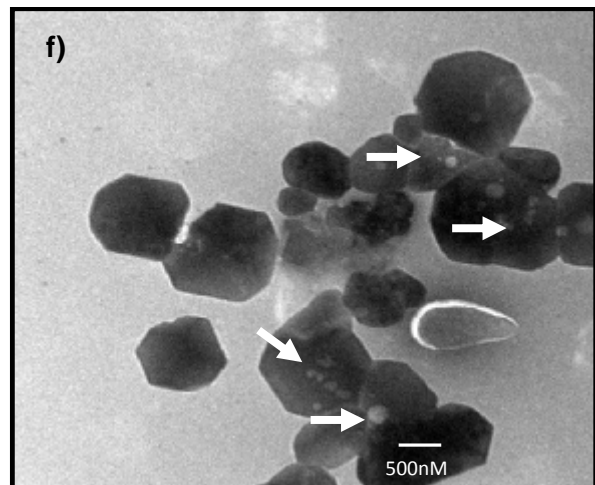
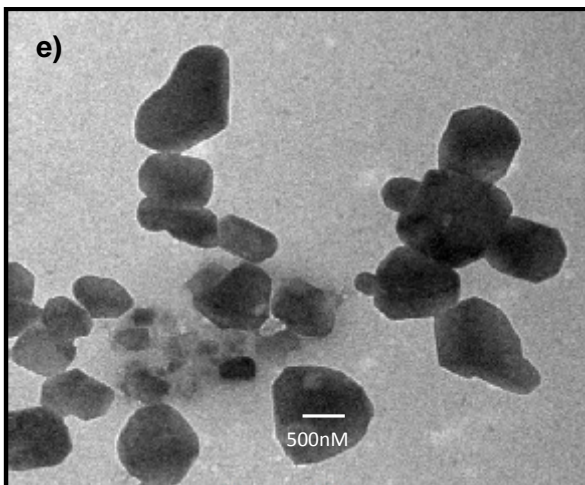
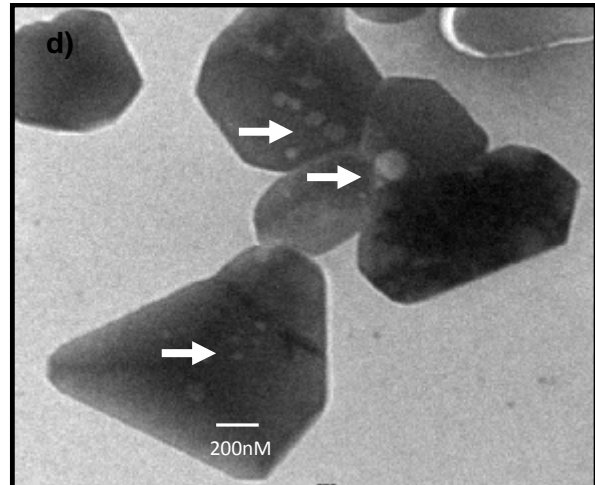
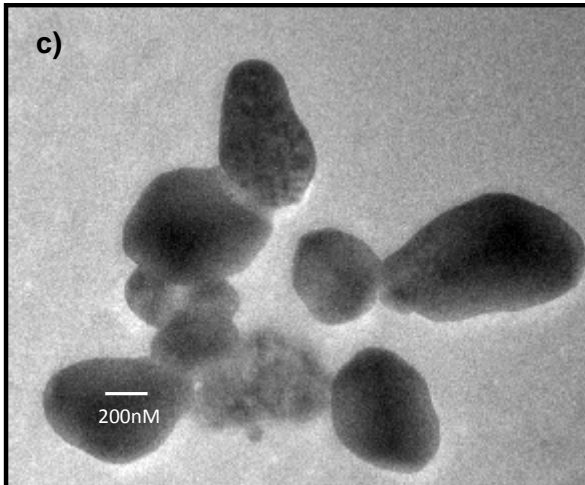
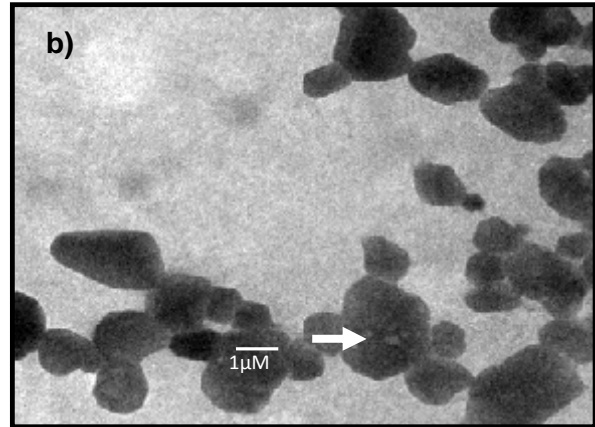
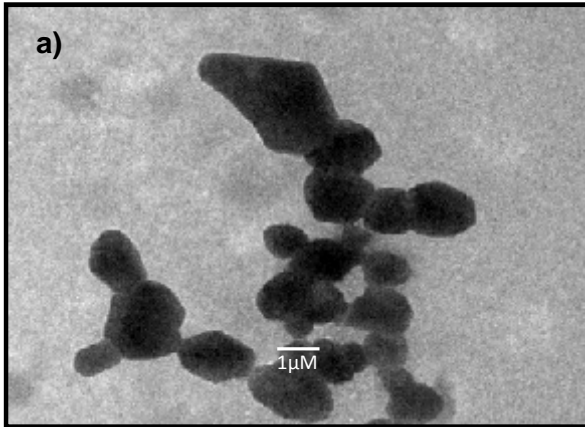
According to Sharma *et al.* (2013), when murine B-lymphocyte cell line (CH12.LX) was treated with citrate stabilized 10 nm gold nanoparticles (Au-NPs). TEM image demonstrated that Au-NPs could pass through the cellular membrane and therefore could interact with intracellular components.

The present study reports of TEM images of different magnifications of 4 μM AgNO₃ treated cultured PBLs at 24 hours are presented in Plate 4.

Plate 4
TEM Images of Cultured PBLs treated with 4 μM AgNO_3 for 24 Hours

Control

4 μM AgNO_3 treated PBLs



TEM images of PBLs grown for 24 hours in presence of zero (Control) and 4 μM treated AgNO_3 . Arrows show electron dense nanosilver precipitates.

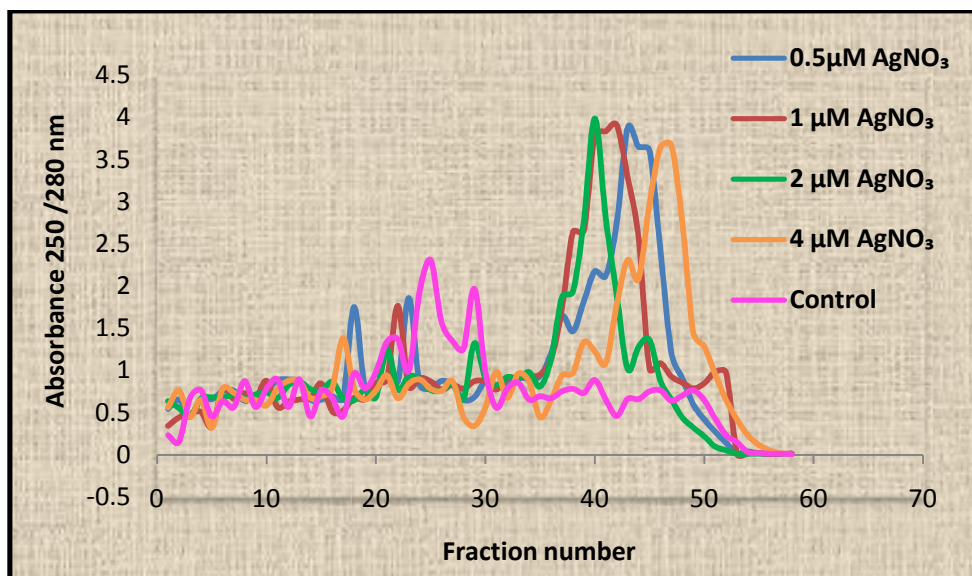
4.3.4 Isolation of metallothioneins from peripheral blood lymphocytes treated with different concentrations of AgNO₃

Peripheral blood lymphocytes (PBLs) were isolated from selected voluntary healthy donors (n=3). Isolated PBLs were then divided into 5 aliquots and cultured in RPMI 1640 medium. Supernatant of the PBLs lysate after 24 hours was eluted in sephadex G-75 gel filtration column at the rate of 15 ml/hour as discussed in Phase II of the study (Section 4.2.1).

Absorbance at 250 nm/280 nm ratio more than 1 indicated the presence of mercaptide bonds. In the present study, absorbance ratio at 250 nm/280 nm was recorded in 58 fractions collected from lysates of PBLs treated with different concentrations of AgNO₃ with the volume of 1.5 ml. Fractions 18, 21, 23 and 36-47 in 0.5µM AgNO₃ treated, fractions 22 and 37- 46 in 1 µM AgNO₃ treated PBLs, fractions 29 and 36–45 in 2 µM AgNO₃ treated, fractions 39 – 50 in 4 µM AgNO₃ treated and fractions 21-29 of control PBLs indicated the presence of mercaptide bonds. These fractions were considered as MTs. The absorbance ratio (250 / 280 nm) of each fraction of control and different concentrations of AgNO₃ treated PBLs is shown in Figure 21.

Figure 21

Profiles of Sephadex G-75 Column Elution Fractions from Cultured Peripheral Blood Lymphocytes showing the Absorbance 250/280 nm



The present study results could be supported by the work of Suzuki *et al.* (1998). Cadmium binding proteins were isolated from the homogenate of rat testis through sephadex G-75 gel filtration column. Further, these cadmium binding protein fractions were considered as MT in the flow through fractions between 48 and 56.

Florianczyk (2003) isolated the metallothioneins from rabbit liver cytosol which was initially heat treated to remove the heat liable proteins and then the cytosol was applied to a gel filtration column (Sephadex G-75). Metallothioneins content in each eluted fraction was measured by them by cadmium hemoglobin affinity assay using the cadmium isotope (^{109}Cd) which indicated the presence of MTs in the low molecular weight fractions.

The elution profile of MT from crude extract of Swine kidneys by gel permeation column showed two major peaks I and II which indicated the presence of high molecular weight proteins (200 to 24 kDa) and low molecular weight proteins (< 24 kDa and MT - 6.5 kDa) respectively. García-rico *et al.* (2002) also revealed a positive correlation between MTs and each of the exposed metals (Cu, Zn and Cd).

A case study was illustrated by Falnoga *et al.* (1998) in which metallothionein-like protein (MT-LP) was extracted from brain cerebellum (nucleus dentatus) of a mercury mine employee by Sephadex G-75 gel filtration column. Their findings revealed that MT in nucleus dentatus could be involved in copper metabolism and in short term heavy metals (Pb and Hg) detoxification.

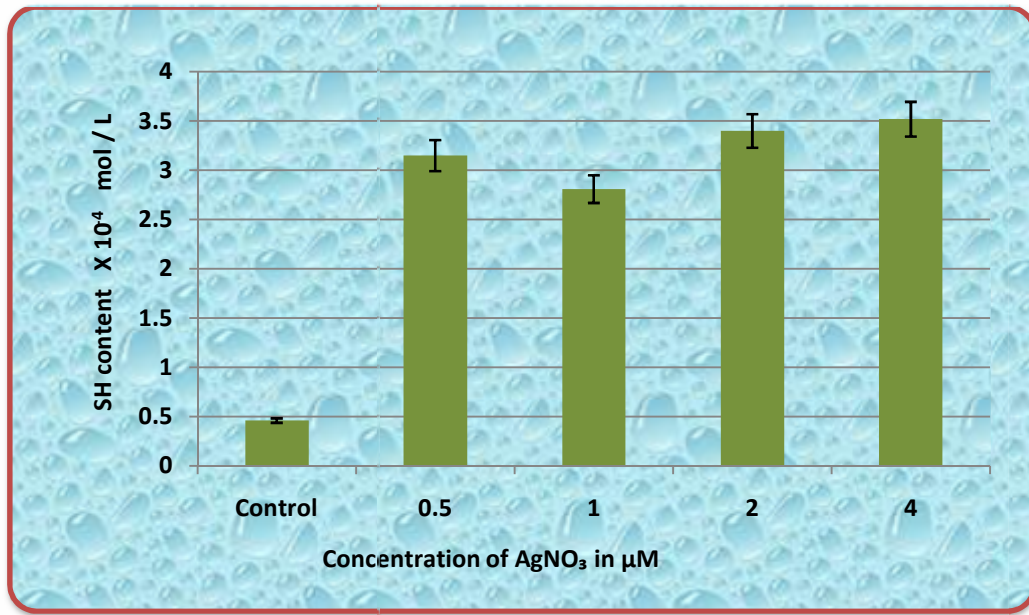
4.3.5 Sulfhydryl content of isolated metallothioneins from cultured lymphocytes

Fractions with 250/280 nm absorbance ratio more than 1 were pooled together, concentrated and assayed for sulfhydryl content of isolated metallothioneins.

Figure 22 features the level of sulfhydryl content of isolated metallothioneins in PBLs treated with different concentrations of AgNO_3 (0.5 μM , 1 μM , 2 μM and 4 μM).

Figure 22

Sulfhydryl (-SH) Content of PBLs treated with Different Concentrations of AgNO_3



Thiols (-SH) have a central role in coordinating antioxidant defense network in biological systems (Sen and Packer, 2000). The most significant characteristic of the sulfhydryl or thiol group is its tightly binding capability to metal ions (Hynek *et al.*, 2012).

MT is low molecular mass protein with unusual content of high cysteine residues that allows it to coordinate multiple metal ions in the form of metal-thiolate clusters. It is intended to play a specific role in the transport and storage of the vital metal ions (Cu and Zn) and to contribute in the detoxification of physiologically harmful metal ions (Cd and Hg). Moreover, the high sulfhydryl contents of this protein and its induction by certain cellular stress conditions propose a function in the direct scavenging of cell damaging reactive oxygen species such as OH[•] and O₂^{•-} resulting in disulfide bridge formation of the cysteine residues (Vasak and Hasler, 2000; Cobbett and Goldsbrough, 2002; Freisinger, 2008 and Maret, 2011).

In the present study, sulfhydryl content of isolated MTs in PBLs treated with different concentrations of AgNO₃ (0.5μM, 1μM, 2μM and 4μM) was found to be increased significantly ($p < 0.05$) compared to control group. But there were no significant differences ($p > 0.05$) noticed in the sulfhydryl content of isolated MTs among the AgNO₃ treated groups.

The elevated levels of MTs determined by sulfhydryl contents in the AgNO₃ treated groups were observed in the present study which might be induced in response to metal exposure and it might be actively involved in protection against oxidative stress. Similar result was also observed in Phase II study as discussed in section 4.2.2.

Supernatant of TE671 cells was chromatographed by Zhu *et al.* (2010) over Sephadex G-75 in order to quantify the thiol and Zn²⁺ content of MT. A discrete MT peak was determined by 5,5'-dithiobis (2-nitrobenzoate) or DTNB and Zn²⁺ analyses enclosing chromatographic fractions between 35 to 45. The study also indicated that MT content was clearly boosted when TE671 cells were pre-incubated with 80 µM Zn²⁺ for 24 hours.

4.3.6 Free radical scavenging activity of metallothioneins from lymphocytes exposed to different concentrations of AgNO₃

Free radicals are common outcome *in vivo* in cellular metabolism. They can be over produced in pathological conditions, causing oxidative stress. In-built antioxidant system of body plays its crucial role in prevention of any loss due to free radicals. However, imbalanced defense mechanism of antioxidants, overproduction or incorporation of free radicals from environment to living system leads to serious problems to living being (Uttara *et al.*, 2009).

The protective and defensive roles of MT are related to the stimulation of this protein during stress conditions or by the existence of toxic agents. The exposure of living being to toxic factors namely heavy metals induces MT expression in different tissues. Thus this protein could be used for exposure assessment in mammals and environmental monitoring of pollution from various metals (Dabrio *et al.*, 2002).

Several studies have been suggested that sulfhydryl group containing proteins participate in protecting against radical induced damage (Rhee *et al.*, 1994; Flora *et al.*, 2008; Flora, 2009). Several researchers used cultured cells and intact animal models to provide further evidence supporting the antioxidant function of MT (Du and Yang, 1994; Yang *et al.*, 1994; Hart *et al.*, 1995).

A raising attention has been focused on the role of MT as a radical scavenger with of its high thiol content that participates in the formation of diamagnetic metal-thiolate clusters (Sato and Bremner, 1993 and You *et al.*, 2002).

Administration of agents that enhance the production of reactive oxygen species (ROS) seemed to increase the level of MTs from 20 to 30 folds in mice. This indicated its involvement in protection against oxidative stress (Bauman *et al.*, 1991). Blood cells and cell lines were also found to produce MTs under numerous circumstances (Pauwels *et al.*, 1994; DeMoor *et al.*, 2001).

It was proved that the high density of MT's thiolate groups reacts with and protects cells from a variety of reactive electrophilic molecules including oxidants such as hydrogen peroxide, hydroxyl and superoxide radicals (Petering *et al.*, 2009; Viarengo *et al.*, 2000). MTs play radical scavenging activity that includes direct interception of free radicals or chelation of transition metal ions acting in radical generation via Fenton-like reaction. Hence in the present study an attempt was made to understand the association between MTs and ROS inhibition in different concentrations (0.5µM, 1 µM, 2 µM and 4 µM) of AgNO₃ treated peripheral blood lymphocytes. Free radical scavenging activity of sulfhydryl enriched protein fraction (MTs) from AgNO₃ treated peripheral blood lymphocytes is shown in Table 19.

Table 19

Free Radical Scavenging Activity of Sulfhydryl enriched Protein Fraction from Peripheral Blood Lymphocytes treated with AgNO₃

Concentration of AgNO ₃ (µM)	Sulfhydryl (-SH) Contents (x 10 ⁻⁴ Mol/L)	Per cent Radical Scavenging Activity			
		ABTS	DPPH	H ₂ O ₂	Superoxide
Control	0.46 ± 0.22	-	-	-	-
0.5	3.15 ± 0.63	33 ± 1.6	31 ± 2	18 ± 2.3	44 ± 3.4
1.0	2.81 ± 0.65	33 ± 0.4	32 ± 1	19 ± 0.9	44 ± 3.3
2.0	3.40 ± 0.83	37 ± 0.8	38 ± 0.6	21 ± 1.5	53 ± 1.3
4.0	3.52 ± 0.99	38 ± 0.4	45 ± 0.6	23 ± 0.9	54 ± 2.9

The values are Mean ± Standard Deviation of triplicates

ABTS, DPPH, H₂O₂ and Superoxide radicals scavenging activity

The ABTS radical is reactive towards most of the antioxidants including phenolics, thiols and Vitamin C (Walker and Everette, 2009). ABTS is a protonated radical which has characteristic absorbance maximum at 734nm and it decreases with the scavenging of proton radicals (Mathew and Abraham, 2006; Adedapo *et al.*, 2008).

The spectrophotometric method of DPPH radicals scavenging activity has been one of the most commonly used methods to estimate the antioxidant activity (Matkowski and Piotrowska, 2006). The DPPH radical was widely used in the model system to investigate the scavenging activities of several natural compounds (Huang *et al.*, 2004).

Hydrogen peroxide is unstable and readily crosses the cell membrane along with other ROS. It undergoes Fenton reaction and initiates lipid peroxidation. Superoxide has the potential to activate biological molecules and causes oxidative damage to cells and tissues (Uttara *et al.*, 2009).

Comparison of ABTS, DPPH, H₂O₂ and Superoxide Radicals Scavenging Potential of Metallothioneins in the present study with earlier findings of researchers is presented in Table 20.

According to the findings of the present study it could be suggested that cysteine residues in MTs have antioxidant properties which might involve in antiradical activity. Moreover, maximum scavenging effect of MT on superoxide radical and least scavenging effect on hydrogen peroxide were observed from the study.

The present study finding is in agreement with the reports of Fliss and Menard (1992), indicating that MT was an extremely efficient and effective reactant with superoxide anion and hydroxyl radical, but it was less reactive with hydrogen peroxide.

4.3.7 Quantification of isolated metallothioneins from cultured lymphocytes by HPLC

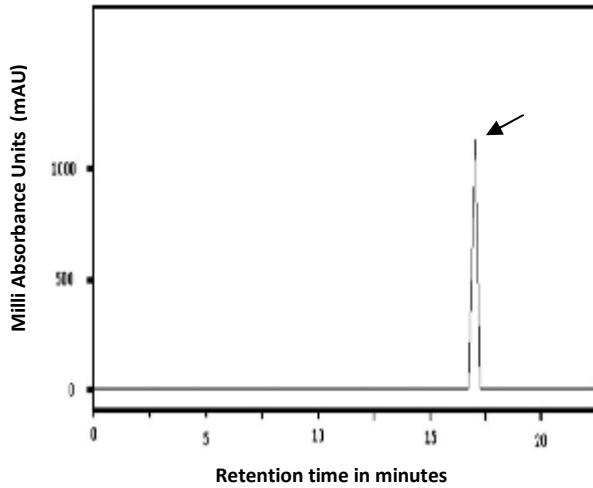
Figure 23 presents the details of high performance liquid chromatography (HPLC) spectra of metallothionein from cultured PBLs treated with minimum (0.5 μ M) and maximum (4 μ M) concentrations of AgNO₃ and control PBLs.

In the present study, the retention time for horse kidney metallothionein standard and AgNO₃ treated groups (0.5 and 4 μ M) were 17.36, 17.39 and 17.40 minutes respectively as analyzed by HPLC (Figure 23a, c and d). Cultured control PBLs did not show MT peak in the HPLC profile (Figure 23b).

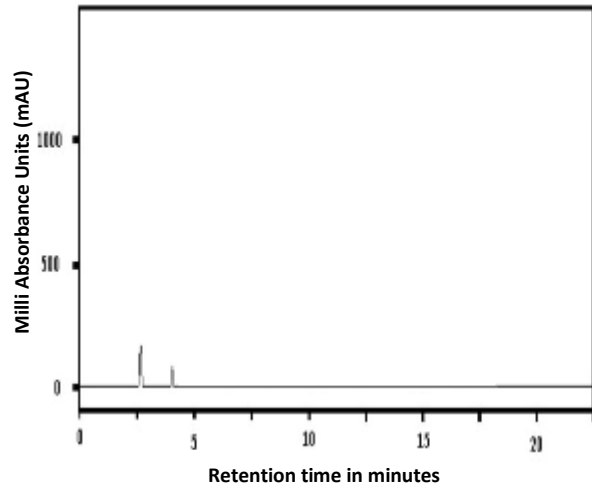
MT in the samples was estimated to be 45.86 % and 47.11 % in 0.5 μ M and 4 μ M AgNO₃ treated cells respectively. This result revealed an elevation of metallothionein in cells treated with both the concentrations (0.5 and 4 μ M) of AgNO₃ and provided evidence that MTs could be induced by metal exposure.

Figure 23
HPLC Spectra of Control and AgNO₃ Exposed Cultured Lymphocytes after 24 Hours

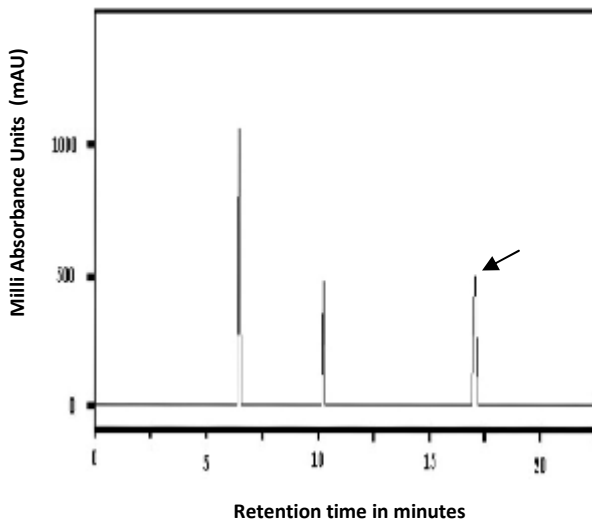
a) Standard (Horse kidney MT)



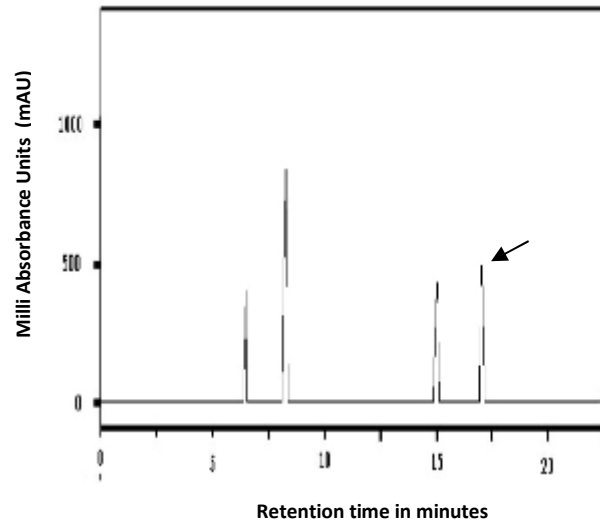
b) Control (PBS treated PBLs)



c) PBLs treated with 0.5 μM AgNO₃



d) PBLs treated with 4 μM AgNO₃



(→) Arrow makes point to MT peaks

Caco-2, human cell lines (CL11) were treated with radio labeled zinc that showed presence of MT in the HPLC fractions and the levels of Zn-MT were found to increase with increase in zinc concentration as reported by Moltedo *et al.* (2000)

MT was extracted and separated by HPLC from placentas saturated with cadmium chloride in smokers and non-smokers. Two peaks were obtained and eluted at 6 and 12.5 minutes corresponding to MT-1 and MT-2 respectively. MT present in both peaks was identified by western blot analysis using a monoclonal antibody directed against MT-1 and MT-2. In placentas of smokers, level of MT-2 was increased by seven fold compared to non-smokers. However, no change in MT levels was noticed in smokers and non smokers as detailed by Ronco *et al.* (2006).

According to Jin *et al.* (1993), MT in rat liver was separated by Superdex 75 high performance gel filtration column and was determined by HPLC. MT was detected by a UV spectrophotometric detector at 250nm which showed retention time of 14.3 minute.

4.3.8 FTIR spectra of Sephadex G-75 column eluted sulfhydryl group enriched protein fraction from AgNO₃ treated lymphocytes

In the present study, FTIR spectra of MT fractions were monitored to study changes in the functional groups of isolated MTs induced by metal ions. The IR spectra were measured between wavenumber 4000 and 400 cm⁻¹. The FTIR spectra of MTs from lymphocytes treated with minimum and maximum concentration of AgNO₃ (0.5μM and 4μM) were compared with control to analyze the presence of stress factors produced during metal exposure.

Figure 24 a, b and c present the details of FTIR spectra of MTs from cultured PBLs of control, 0.5μM AgNO₃ and 4μM AgNO₃ treated cells.

Figure 24 a

FTIR Specum of Sephadex G- 75 Column eluted Sulfydryl group Enriched Protein Fraction from PBS Treated PBLs after 24 Hours (without AgNO₃ treatment)

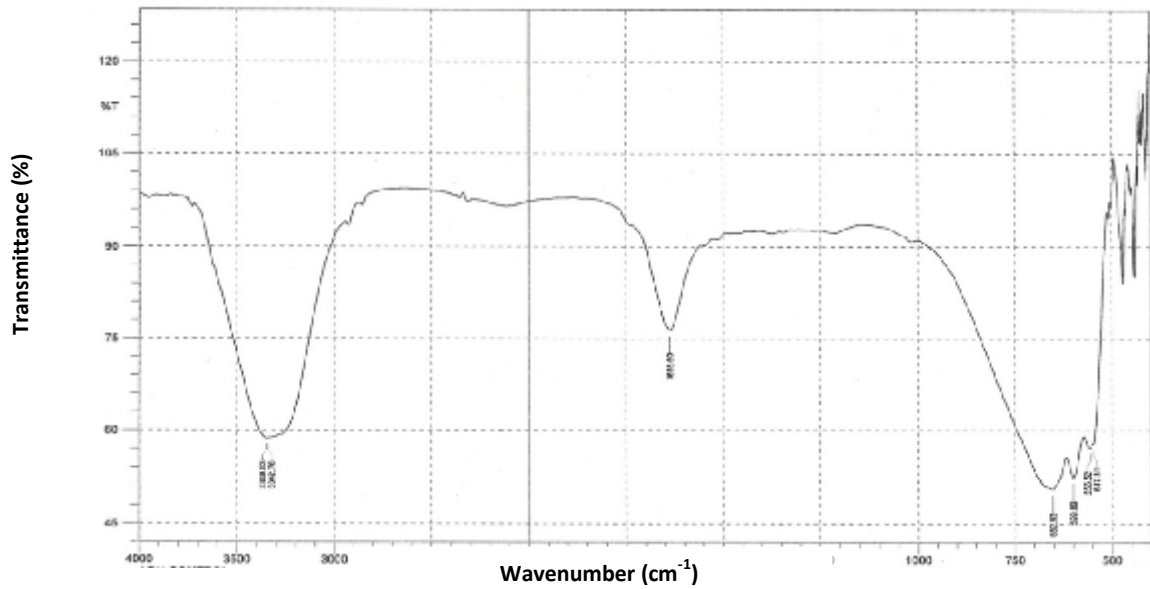


Figure 24 b

FTIR Specum of Sephadex G- 75 Column eluted Sulfydryl group Enriched Protein Fraction from 0.5μM AgNO₃ Treated PBLs after 24 Hours

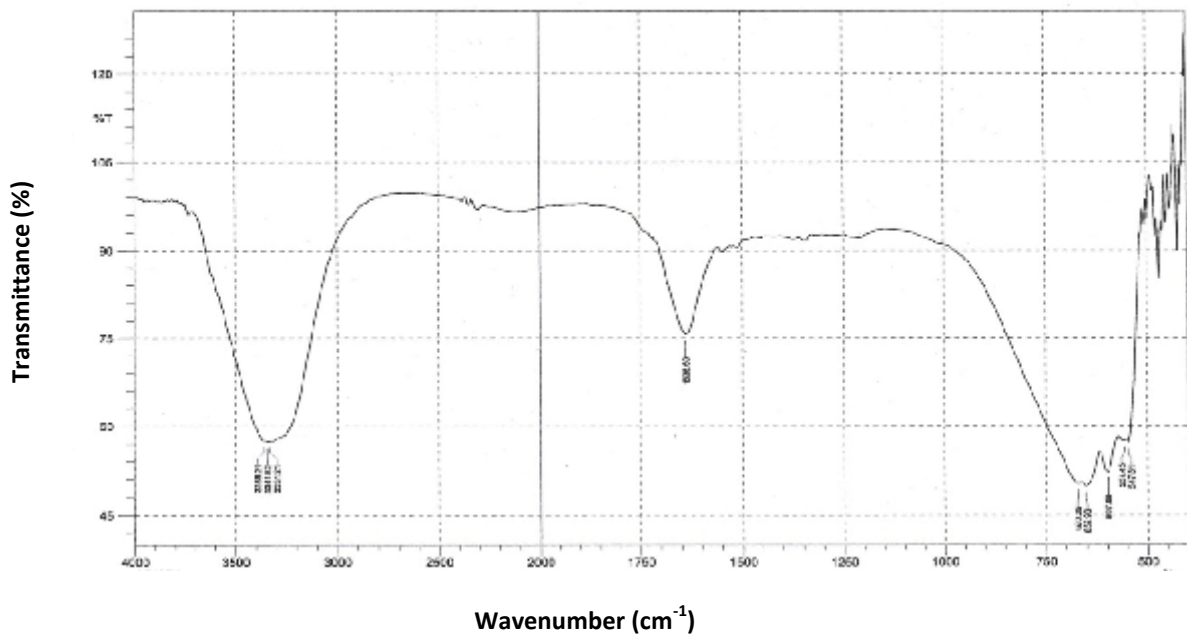


Figure 24 c

FTIR Specum of Sephadex G- 75 Column eluted Sulfydryl group Enriched Protein Fraction from 4 μ M AgNO₃ Treated PBLs after 24 Hours

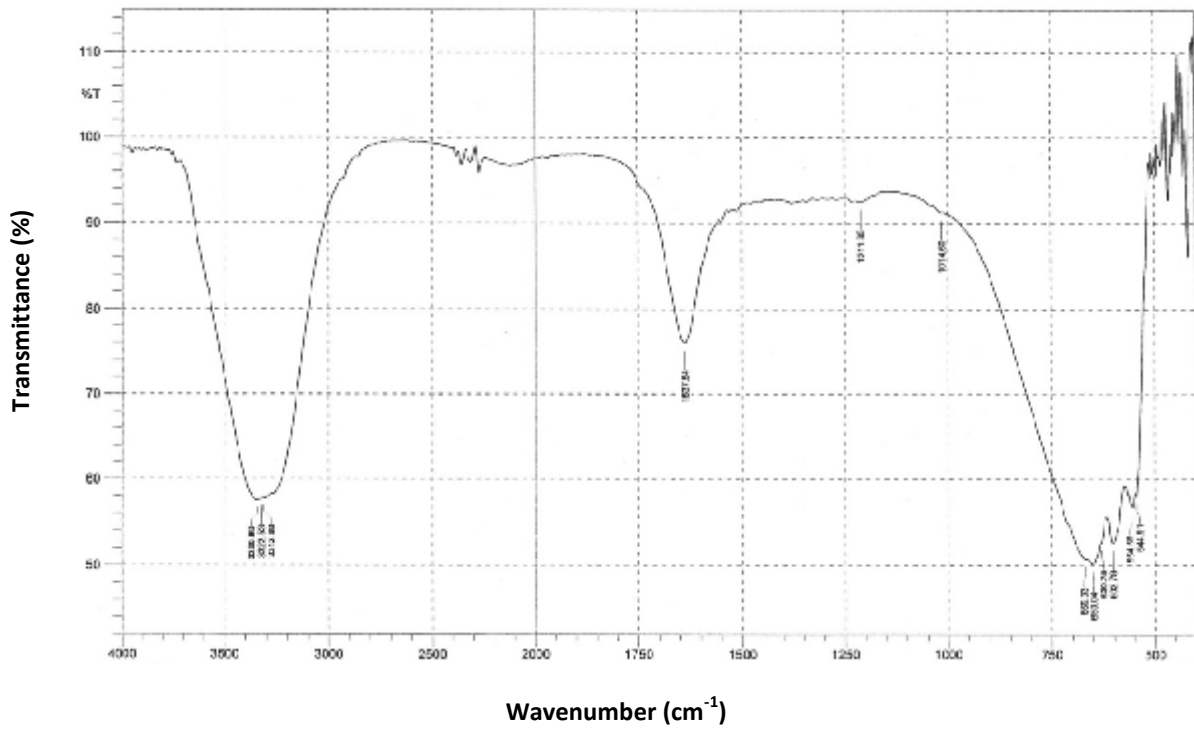


Table 21 illustrates the interpretation of FTIR Spectra of MT from cultured PBLs treated with 0.5 μ m and 4 μ m AgNO₃.

Table 21

Interpretation of FTIR Spectra of MT from cultured PBLs on treatment with 0.5 μ M and 4 μ M AgNO₃

Observation and Interpretation	
Present study report	Earlier reports
<p>Broad bands at 3358.21 cm⁻¹, 3341.82 cm⁻¹ and 3331.21 cm⁻¹. MT from 4 μM AgNO₃ treated cultured PBLs provided broad peaks at 3339.89 cm⁻¹, 3322.53 cm⁻¹ and 3312.88 cm⁻¹. MT from control showed peaks at 3349.53 cm⁻¹ and 3342.78 cm⁻¹</p> <p>Represent O-H and N-H stretching vibrations of protein</p>	<p>Broad bands around 3330 - 3370 cm⁻¹ of proteins (Wolkers <i>et al.</i>, 1998).</p> <p>Represent O-H and N-H stretching vibrations that are mainly generated by protein</p>
<p>No bands by MT's of cultured PBLs treated with 0.5μM and 4μM AgNO₃ and controls at 2600-2500 cm⁻¹</p> <p>Proof for absence of free thiol (-SH) or sulfhydryl moiety in MTs</p>	<p>Free cysteine in MTs can be evidenced by -SH stretching band at 2600-2500 cm⁻¹ wavenumber (Nemeth, 2007).</p> <p>Proof for the presence of free thiol (-SH) or sulfhydryl moiety in MTs and no other groups give rise to bands in this region</p>
<p>Bands at 1637.64 cm⁻¹, 1638.60 cm⁻¹ and 1638.60 cm⁻¹ in control, 0.5μM and 4μM AgNO₃ treated PBLs</p> <p>Bands indicating the presence of amide - I group (C=O stretch) in MT</p>	<p>Amide - I region of proteins indicating bands at 1700-1600 cm⁻¹ (Surewicz <i>et al.</i>, 1993; Nishina <i>et al.</i>, 2007).</p> <p>Corresponds to C=O stretching vibrations of polypeptide which can find out changes of the overall protein conformation and content</p>

Table 21 (Contd...)

Observation and Interpretation	
Present study report	Earlier reports
<p>No bands by MT's of cultured PBLs treated with 0.5μM and 4μM AgNO₃ and controls at 1656 cm⁻¹ and 1621 cm⁻¹</p> <p>Proof for absence of secondary structure (α turn and β sheet) of MT</p>	<p>bands at 1656 cm⁻¹ and 1621 cm⁻¹ (Wei <i>et al.</i>, 2009)</p> <p>Represent the α turn and β sheet of secondary structure of protein</p> <p>Conformational dynamics and structural properties of Antarctic fish (<i>Notothernia coriiceps</i>) and mouse MTs were studied and observed no bands at 1656 cm⁻¹ and 1621 cm⁻¹ region (Capasso <i>et al.</i>, 2002).</p> <p>Fish and mouse MTs lack secondary structural elements such as α helices and β sheet</p>
<p>Band at 1211.35 cm⁻¹ in 4μM AgNO₃ treated and no such peak in 0.5μM AgNO₃ treated PBLs and control</p> <p>Bands might indicate the presence of amide – III (C-N stretching and N-H in plane) group in MTis</p>	<p>Bands at 1320 – 1205 cm⁻¹ of amide III group of protein (Ozaki <i>et al.</i>, 2003; Zhao, 2008)</p> <p>Presence of C-N stretching and N-H in plane of amide – III group in protein</p>
<p>Bands at 652.93 cm⁻¹ for control, 652.83 cm⁻¹ for 0.5 μM AgNO₃ treated PBLs and 650.04 and 630.75 cm⁻¹ for 4μM AgNO₃ treated PBLs</p> <p>Presence of thioethers (CH₃ – S) stretch in MT is suggested</p>	<p>Bands at 660 - 630 cm⁻¹, 620 – 600 cm⁻¹ and 705 – 550 cm⁻¹ in protein (Coates, (2000).</p> <p>Presence of thioethers (CH₃ - S) stretch, disulfides (S-S) stretch and disulfides (C-S-S-C) stretch in protein</p>

Table 21 (Contd...)

Observation and Interpretation	
Present study report	Earlier reports
<p>Bands at 599.89 cm⁻¹ of controls, 597.96 cm⁻¹ for 0.5 μM AgNO₃ treated PBLs and 602.78 cm⁻¹ for 4μM AgNO₃ treated PBLs</p> <p>Presence of disulfides (S-S) stretch in MT is suggested</p>	<p>No earlier reports available</p>
<p>Bands at 555.52 and 547.81cm⁻¹ of controls, and 670.29,557.45 and 547.81 cm⁻¹ for 0.5 μM AgNO₃ treated PBLs and 669.33, 554.56 and 544.91 cm⁻¹ for 4μM AgNO₃ treated PBLs</p> <p>May be due to presence of disulfides (C-S-S-C) stretch in MT</p>	<p>No earlier reports available</p>

These results showed that there are more number of disulfide bonds present in MTs in the PBLs treated with 0.5 and 4μM AgNO₃ compared to control. This might support that metals actively bind to sulfur atoms of MTs through mercaptide bonds. However, it could be suggested that metal free MTs take a random coil structure consisting of disulfide bonds that is created for structural stability of proteins.

Domenech *et al.* (2007) observed that IR spectrum of MT (Cd – QS MT) proved cadmium (II) binding abilities of recombinant cork oak (*Quercus suber*), where MT exhibited 8 and 10 components in the 1320 – 1205 cm⁻¹ and 1720 – 1580 cm⁻¹ wavenumber respectively resulting from the involvement of different secondary structural elements. But α helix, β turn and β sheet segments in the study seemed to be less and negligible.

MTs do not contain disulfide bonds in the primary structure and do not form alpha helices or beta sheets as secondary structures. Hence metal free MTs take a random coil as three dimensional structures to retain their stability as suggested by Stillman (1995). FTIR spectra of MTs in the present study might indicate that they do not contain secondary structure (α helix and β sheet) and also consist of disulfide bond in MT of both the AgNO_3 treated cells (0.5 and $4\mu\text{M}$) proving that the isolated MTs are more flexible and take random coil structure. Similar results were also observed in the Phase II of the study (discussed in Section 4.2.5) in FTIR spectral analysis of isolated MTs from PBLs of jewellery unit workers.