REVIEW OF LITERATURE

The literature pertaining to the present study on "***In vivo* and *In vitro* Studies on the Expression of Metallothioneins in Response to Silver Exposure among Jewellery Unit Workers**" is reviewed under the following headings:

2.1 Scenario of Heavy Metal Pollution

Environment has been contaminated with heavy metals as a consequence of rapid urbanization, industrialization, growth of human population, enormous increase in vehicular traffic, mining activities and use of chemical fertilizers and pesticides (Shatalov *et al*., 2011). Some of the oldest human diseases could be traced to heavy metal poisoning associated with its handling. Even with the present recognition of the hazards of heavy metals, the incidence of intoxication remains significant and the need for effective therapy remains high (Bryson, 1996).

2.2 Silver Exposure in Jewellery Units

In jewellery industry, pure gold is alloyed with copper, zinc and silver in varying proportions to produce wide range of karat gold in which silver is used in higher concentration during the processing of gold. Silver is classified as a xenobiotic metal having less known physiologic function in the human body.

NIOSH (National Institute for Occupational Safety and Health, 2003) reports that people are potentially exposed to silver in workplace environments. According to industrial applications, jewellery, silverware and the photographic industries were the largest consumers of silver using 40, 31, 22% respectively (Gold Fields Minerals Services, 2004).

During the manufacturing processes of jewellery, formation of fumes and dusts which consist of metals and hazardous compounds is common. Goldsmiths are also known to use potent toxic chemicals like amile nitrates, ferric oxide, aluminum oxide (polishing compounds), ammonium chloride, aniline dyes, cadmium, cadmium bicarbonate, copper sulphate, mercury, potassium cyanide, potassium hydroxide, potassium nitrate, silver, silver nitrate, nitric acid and investment powder (plaster of paris and silica). These chemicals are used for melting, casting, welding and polishing the jewellery.

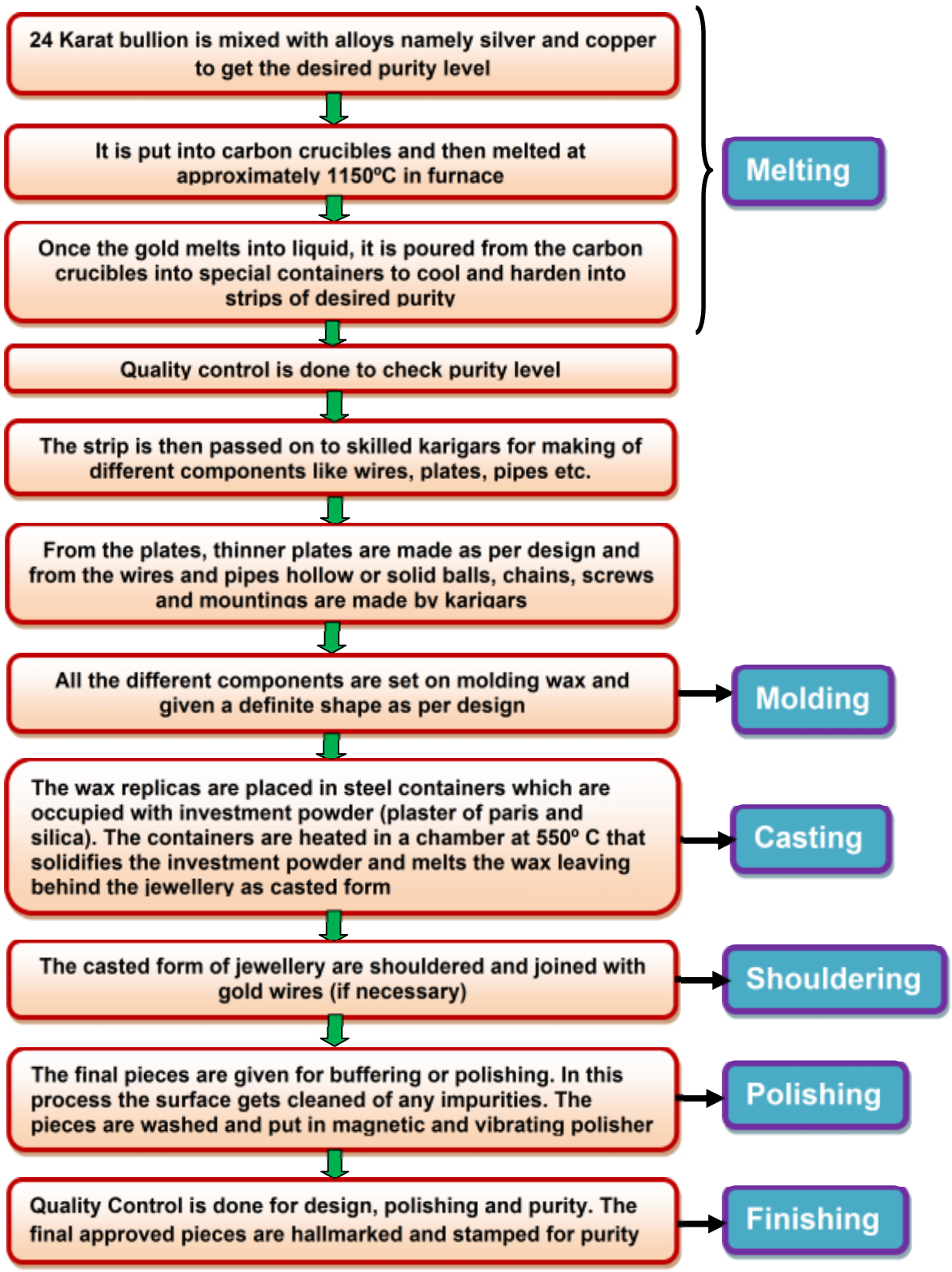
Many of these substances are potentially harmful to human health. Workers in these units inhale, ingest and absorb large amounts of these substances daily over extended periods of time. The major route of silver entry into the body is gastrointestinal tract (ingestion) (Silver, 2003), lungs (inhalation) (Lee *et al.*, 2010) and dermal absorption (Larese *et al.*, 2009). In occupational exposure, respiratory absorption is usually greater than intestinal absorption, but ingestion and dermal absorption are also routes for the entry of silver into the body (Drake and Hazelwood, 2005). Silver absorption by any route is determined by the source of silver contamination, its ability to produce biologically active ions (Ag^+) and duration of exposure (Burrell, 2003).

Figure 1 shows the processes involved in the manufacture of jewellery.

2.2.1 Gold jewellery manufacturing process

Figure 1

Processes Involved in Jewellery Manufacture



2.3 Uptake and Metabolism of Silver in Human Body

During the manufacturing processes soluble, insoluble and metallic silver compounds are inhaled, ingested and absorbed by the workers through various organs. Most of the previous occupational exposure reports showed soluble silver compounds that cause toxic effects at lower concentrations than insoluble and metallic silver (Drake and Hazelwood, 2005).

Experimental and clinical studies have illustrated that metals absorbed into the body interact and compete for binding sites on carrier proteins. These key metal-binding proteins namely metallothioneins initiate protective and detoxification mechanism against the absorbed metals (Lansdown, 1995; Idson, 1978; Lansdown *et al*., 2001).

Absorption

Silver can enter into the body through various portals like dermal absorption (skin), inhalation (lungs and respiratory tract) and ingestion (gastrointestinal tract) of fumes and dusts containing silver compounds. The small nano-sized particles of silver can bind to different proteins and cause phagocytosis mostly in the form of silver protein complex (Ag-MT and Ag-GSH) (Chen and Schluesener, 2008). In human tissues, metallic silver is inert but it ionizes in the moisture, body secretions and fluids to release the biologically active Ag^{+} ions which shows a strong affinity towards sulfhydryl groups and other anionic ligands of proteins, cell membranes and tissue debris (Burrell, 2003).

Armitage *et al*. (1996) revealed that inhaled silver can be absorbed through the wall of alveolus and deposited in lungs. Nearly 10-20% of ingested silver compounds are absorbed through the gastrointestinal tract, mainly by the duodenum and small intestine. Some of the studies have been reported about the absorption of ingested metal through skin, but the level of absorption may depend on the damage to epithelium layer being present (Boosalis *et al*., 1987).

Circulation and metabolism

Silver is absorbed and ingested through mucus membranes of the buccal cavity and proximal aspects gastrointestinal tract from contaminated food and water of unhygienic practice of workers in the industry (Fung and Bowen, 1996). When silver

enters the body, it can be absorbed in the cellular environments and it makes adhesive interactions with biological molecules namely albumin and metalloproteins which can be translocated by blood stream within the human body (Jongerius and Jongeneelen, 1992).

Silver and silver compounds or biologically active ions (Ag^+) are deposited in various tissues throughout the body and they are mainly accumulated in liver, spleen and kidneys (Armitage *et al*., 1996). Some of the other areas of human body includes eyes, internal organs and sun exposed areas most likely to become pigmented due to silver exposure (Juberg and Hearne, 2001).

Metallic silver is not soluble in aqueous solutions nor is it readily solubilised by any physiological mechanisms (Grabowski and Haney, 1972; Weir, 1979). Hence it is poorly absorbed after exposure and is more likely to be excreted by the body (Health and Safety Executive, 1998). But some of the studies indicated that metallic silver is biologically inert, however it gets gradually ionized in moisture, body secretions and fluids to release Ag^+ which is absorbed through skin, respiratory tract, gastrointestinal tract and uriogenital tract (Wan *et al*., 1991; Burell, 2003).

Silver exposure has been investigated in a range of laboratory animals which showed that absorbed silver is metabolized in soft tissues leading to changes resembling to argyria (pigmentation of skin) and argyrosis (pigmentation of eyes) (Fung and Bowen, 1996).

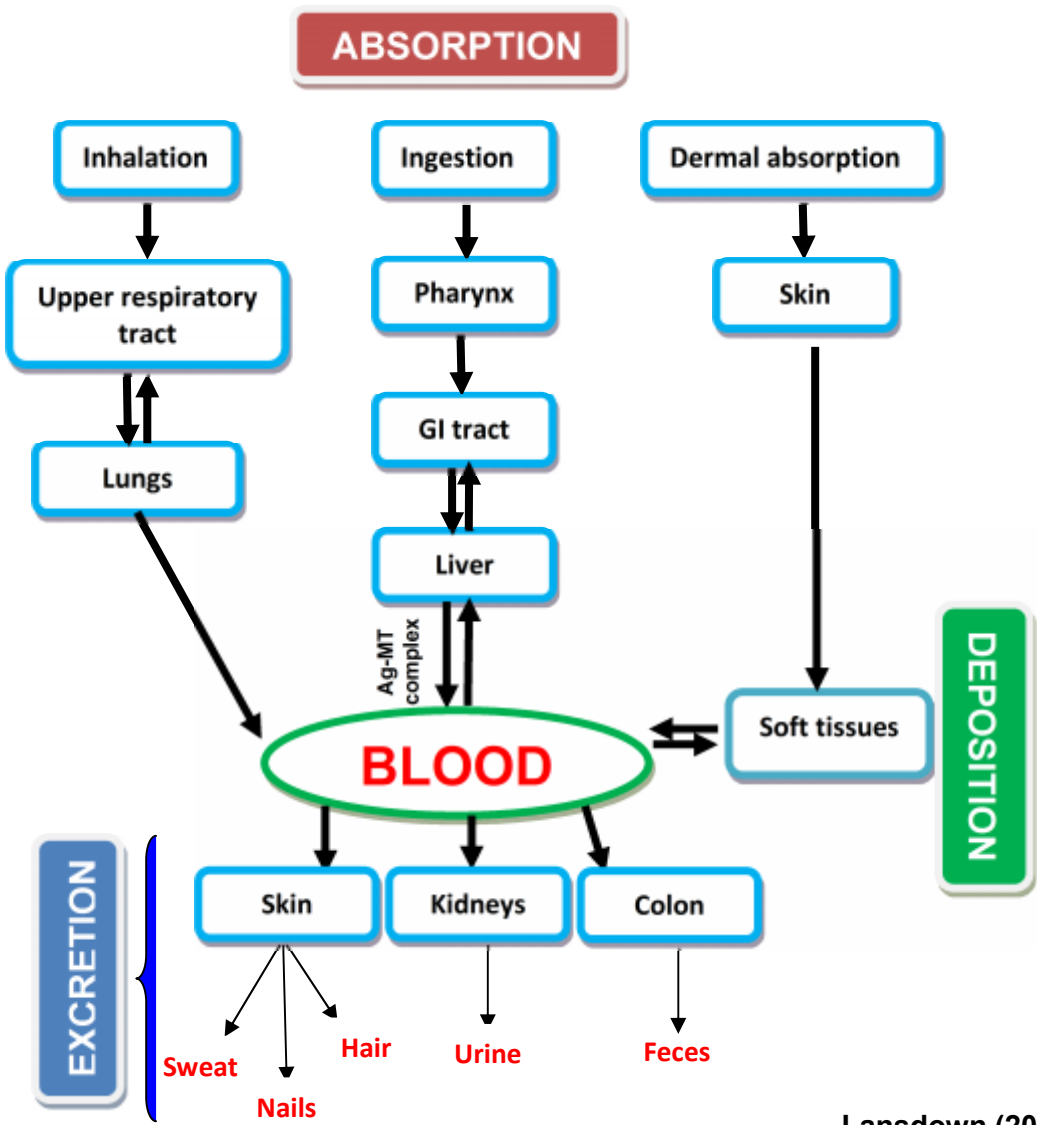
Excretion

The silver protein complex is metabolized in liver and mostly excreted via urine and feces (*Baldi et al., 1988).* Elimination of silver from the body is primarily (90 %) through fecal excretion and minor quantity via urine and hair (Juberg and Hearne, 2001). But some of the unmetabolized or biologically active ions (Ag^+) are deposited in tissues or circulated in the biological system which becomes toxic factors that cause oxidative damage to the cells (Lansdown, 2010; Lansdown, 2007; Manoj and Padhy, 2013).

Figure 2 shows the metabolism of silver in humans

Figure 2

Silver Uptake and Metabolism in Humans



Lansdown (2010)

2.4 Silver Induced Health Effects in Humans and Symptoms of Silver Toxicity

Silver has toxic effects on human health. It can enter into the human body through various portals. Some of the previous studies indicated that Ag^{+} causes early changes in permeability of cell membrane and mitochondrial function (Gopinath *et al*., 2008). Shin *et al*. (2007) demonstrated that silver has toxic effects on proliferation and cytokine expression in peripheral blood mononuclear cells.

According to Xia *et al*. (2006), silver nanoparticles can attach to different tissues and cause potential toxic effects namely producing reactive oxygen species and cell activation. It is more toxic to organs and tissues and gradually lead to cell death. Drake and Hazelwood (2005) revealed that skin, eyes, brain, liver, kidneys, spleen and bone marrow are the principle target tissues for silver deposition following systemic absorption. Sue *et al*. (2001) reported that over exposure to silver can cause accumulation in the skin, blood, liver, spleen, corneas, mucous membranes, kidneys, hair, gingival and nails.

A case study of 29 years old man who accidentally inhaled dust containing ^{110m}Ag and ^{65}Zn was monitored in a minor nuclear reactor incident. Radio labelled silver was monitored in his lungs, urine, and feaces for up to 200 days as reported by Newton and Holmes (1966).

Rosenman *et al*. (1979) conducted a cross sectional study in 30 workers from an industrial plant who were involved in manufacturing of silver nitrate and silver oxide in which 6 individuals had generalized argyria and 20 had argyrosis. Ten of the 30 workers complained of abdominal pain. These symptoms were significantly associated with silver in the blood. Decreased vision at night was complained by 10 workers which was associated with duration of employment.

Moss *et al*. (1979) also studied the same group of workers as Rosenman *et al*. (1979) and found that 27 of 30 workers had suffered burns of the skin from contact with silver nitrate and 11 workers had a history of ocular burns. The most commonly noted abnormal finding was discoloration of conjunctiva and cornea which was directly correlated with duration of exposure. The workers suffered with decreased night vision which was significantly correlated with silver deposition in conjunctiva or cornea and with duration of occupational exposure.

According to Di-Vincenzo *et al*. (1985), absorption and excretion of silver were examined by estimating blood, urine, fecal and hair concentrations from 37 workers occupationally exposed to insoluble silver compounds which were higher in their levels of silver compared to the controls. Westhofen and Schafer (1986) presented a case of a

patient with generalised argyrosis associated with progressive taste and smell disorders, vertigo and hypesthesia by exposure to silver compounds.

Rosenman *et al*. (1987) conducted a study in silver and other metal powders manufacturing company in which 96% and 92%of the workers had elevated urine and blood silver concentrations respectively. Out of 27 workers, 15 workers had upper respiratory irritation namely sneezing, itchy, red, or watery eyes, running nose and sore throat. Eight and 6 workers complained of nosebleeds and decreased night vision respectively. Seventeen workers had conjunctival deposits and 6 had corneal deposits. Moreover, creatinine clearance was significantly depressed and urinary N-acetyl-beta-D glucosaminidase (NAG) was significantly higher in the exposed group than the controls which indicated that renal function was adversely affected by the work place pollutants.

Twenty seven silver reclamation workers exposed primarily to insoluble silver halides were clinically evaluated for silver exposure by Pifer *et al*. (1989) and compared with controls. Mean level of silver in blood of 21 silver reclamation workers was 0.01 mg/ml. Among the exposed group workers only one worker had a detectable level of urinary silver. But silver was not detected in the blood or the urine of the control group. Also silver was quantified in fecal samples of all exposed group workers. Clinical examinations and skin biopsies revealed no sign of generalized argyria and 20 out of 27 silver reclamation workers showed some degree of internal nasal septal pigmentation. But no abnormalities were reported during tests of renal function, pulmonary function and chest radiographs.

A study on two men occupationally exposed to silver for different time periods was conducted by Williams and Gardner (1995). No evidence of argyria or argyrosis was seen in the first worker. But deposition of silver in the nasal or oral mucosa was noticed. Atmospheric concentration of silver was recorded as 0.085 mg/m^3 suggesting that the potential for exposure was significantly above the occupational exposure limit. Blood silver level was estimated as 49 mg/l. In the second case, no evidence of argyria was seen, but argyrosis was evidenced as a gray pigmentation of the conjunctiva. Atmospheric silver concentrations were recorded at levels of 0.03 - 0.17 mg/m^3 and

blood silver concentration was 74 mg/l. This study also proved that soluble form of silver was more toxic than insoluble form of silver.

In a clinical situation, use of a silver impregnated bone cement led to a 1000 fold increase of silver level in acetabular cavity of 103.3 μg L^{-1}, but this increased local and systemic levels of silver were not associated with osteological damage in this patient as reported by Sudmann *et al*. (1994).

According to Sharma *et al*. (1997), daily administration of 50mg silver to 30 healthy volunteers for 20 days led to transitory increases in blood cholesterol, phospholipid, triglycerides, glycaemia and associated enzymes. High hepatocellular silver deposition was evidenced in patients by electron microscopy and the precipitates were inert, lysosomally bound and presumably extruded into bile ducts as a normal physiological process.

A case study was performed by Williams (1999) who reported that a 51 years old man worked as a silver refiner for 7 years in a silver nitrate and silver oxide manufacturing unit. It was proved that air silver level in the work place environment and blood silver level were found to be 0.11–0.17 mg/m^3 and 74 µg/dl respectively and this person had corneal and conjunctival argyrosis.

Greater occupational risk may be experienced by those exposed to inhalation of airborne silver particles at work places. Burrell (2003) studied how nanocrystalline particles (<20 nm in diameter) are dissolved more rapidly in moisture and achieve greater absorption in the body. His study revealed that silver readily dissolved in alveolar fluid which could lead to greater lung volumes. Alveolar macrophages dissolved the large proportion of inhaled silver particles in alveolar moisture and to invoke inflammatory changes.

Nephrotoxic consequences of silver might arise mainly from the tubular reabsorption of silver - protein complexes and were starting a cascade of events leading to cell membrane damage and oxidative stress as reported by Franchini *et al*. (2005).

Clinical and experimental studies demonstrated liver as the principle organ for silver accumulation and elimination which showed transitory changes in certain metabolizing enzymes and proved that silver was a cause for irreversible pathological hepatic damage. But no evidence of advanced argyria was seen in patients with blood silver of >200 μg L^{-1} as reported by Coombs *et al*. (1992) and Trop *et al*. (2006).

Panyala *et al*. (2008) reported that inhalation of silver nitrate dust was causative for bronchitis, squamous metaplasia and pigmentation of respiratory tract resembling anthracosis and siderosis.

2.5 Experimental Studies on Silver Exposure in Animal Models

Environmental Protection Agency of United State (1992), has reported that experimental studies in animal models have shown variations in hepatic management and biliary excretion of silver. Intravenous injection of dilute silver nitrate was associated with biliary excretion patterns of 0.25 μg kg^{-1}/min in rats, 0.05 μg kg^{-1}/min in rabbits and 0.005 μg kg^{-1}/min in dogs. Zelazowski *et al*. (1989) revealed that Ag^{+} ions could bind to MT-1 and MT-2 and it was eliminated innocuously in bile without causing any morphological changes in the animal.

Kim *et al*. (2008) conducted subacute toxicity tests in rats by administering silver nanoparticles (60mm) for 28 days. This study showed that rats did not record any change in their body weight at massive doses of 1000 mg kg^{-1}. But noticeable changes were observed as increased alkaline phosphatase and cholesterol levels at doses of >300 mg kg^{-1} that might reflect functional liver changes in tissue.

Suguwara and Suguwara (2000) emphasized marked interspecies differences in silver metabolism and excretion. Rabbits excreted silver at one tenth of the rate whereas rats and dogs at one hundredth of the rate. Experimental studies in rats have also illustrated that copper and antioxidants namely selenium and vitamin E could influence hepatobiliary transport and retention of silver in the liver. This study also highlighted that copper and silver were well known to interact with MT and ceruloplasmin whereas selenium exhibited a strong tendency to precipitate silver as silver selenide thereby promoting silver retention in the tissue.

Few experimental studies in rodents have reported that low nephrotoxicity of silver in the urinary tract has been confirmed by administration of silver nitrate intravenously or in drinking water. They observed precipitation of silver on basement membranes, arteriolar endothelia and elastic laminae. They also noted high levels of renal selenium sulphur and silver in the precipitates in renal membranes (Berry *et al*., 1995a; Berry *et al*., 1995b).

As in human studies, precipitates of silver sulphide or silver selenide were lysosomally bound, but renal toxicity was not observed in mice at high doses of silver nitrate (65 mg kg^{-1}) daily for up to 14 weeks as reported by Environmental Protection Agency (1992). However, there were strong indications from *in vitro* models that Ag^{+} ions interact with and bind to the hydroxyapatite complex and could displace calcium and magnesium ions (Lansdown, 2009). Other researchers have demonstrated that Ag^{+} ions induce calcium release from the sarcoplasmic reticulum in skeletal muscle by acting on the calcium-release channels and calcium-pump mechanisms, presumably through oxidising sulphydryl groups (Tupling and Green, 2002). But no cases of osteoporosis have been reported by long term ingestion or inhalation of silver or implantation of silver coated or impregnated orthopaedic devices.

Elle *et al*. (2013) showed that exposure to 500 mg kg^{-1} of silver nanoparticles in Sprague Dawley rats resulted in liver damage by the dysfunction of lipid metabolism. Silver nanoparticles lead to significant increase in blood urea nitrogen and creatinine levels in male laboratory mice compared to control group as proved by Dayani *et al*. (2014).

Inadequate proof is available to demonstrate that administration of non- ionisable or ionisable silver compounds in pregnancy is a cause of infertility, impaired foetal growth, or abnormal development in any species. Silver nitrate (1%) administered by intrauterine injection to 13 cynomolgus monkeys between 27 and 43 days of pregnancy seemed to cause early vaginal bleeding and termination of pregnancy but two of seven animals re-mated became pregnant again and delivered healthy offspring as reported by Mc-Cauley *et al*. (1994).

According to Eisses and Kaplan (2005), copper trafficking protein (ctr-1) was found to effectively transport silver into the cells exposed to even low micro molar concentration. $^{110}AgNO_3$ injected rats showed that silver metabolism resembles that of copper with respect to tissue distribution. Silver was not found to inhibit internal copper absorption but it was found to affect copper metabolism as reported by Hanson *et al*. (2001).

2.6 Cell Culture Studies for Silver Exposure

Cell culture systems have been developed in recent years as inexpensive mode of investigating intracellular metabolism of xenobiotic materials and mechanisms of cellular toxicity. A large number of *in vitro* toxicity studies have been published in recent years on cytotoxic effects of metallic silver, silver sulphadiazine or other silver compounds. But observations in cultured fibroblasts, keratinocytes, and other human cell lines seemed to reflect the ability of Ag^+ ions to interact with sulphydryl groups in protein and enzymes associated with cell membranes leading to denaturation, structural damage and mitochondrial dysfunction as seen in bacterial and fungal cells (Lansdown, 2006).

According to Cortizo *et al*. (2004), silver wire treated osteoblasts cultured cells failed to report cell cytotoxicity after 48 hours, but showed reduction in alkaline phosphatase activity. Mc-Cauley *et al*. (1989) reported that short term exposure of silver sulphadiazine to human diploid fibroblasts and fresh human donor dermal fibroblast cells showed impaired proliferation associated with marked changes in cell morphology including cytoplasmic deterioration and degeneration of nuclei and cell organelles.

Silver is absorbed into cultured cells by a pinocytic mechanism as in bacteria and fungi which can be interacted with cytoplasmic proteins leading to cell death. According to Arora *et al*. (2008), cultured cells exposed to silver particles at 6.25–50 μg mL^{-1} showed altered cell shape, evidence of oxidative stress and increased lipid peroxidation.

2.7 Metallothionein Isoforms and their Role in Metal Detoxication and Homeostasis

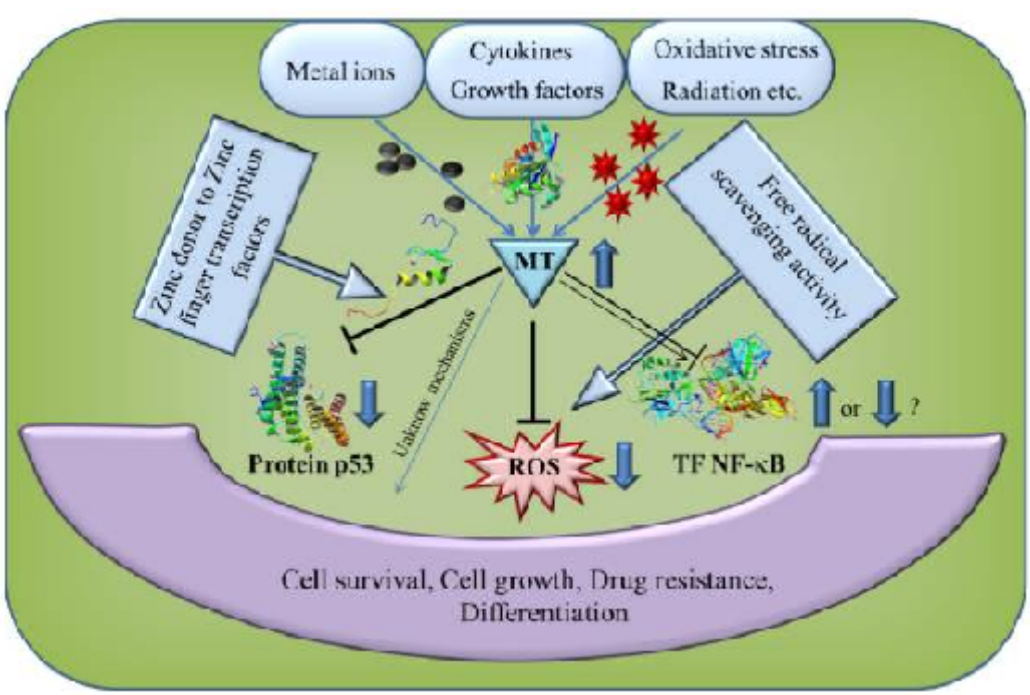
2.7.1 Metallothioneins

Metallothionein (MT) was first isolated from the cortex of horse kidney as a cadmium binding protein in 1957 (Margoshes and Vallee, 1957). Kagi and Vallee in 1960 and Kojima *et al*. in 1976 reported about this protein as low molecular weight (7 kDa), cysteine rich (30%), heat stable and metal binding protein. There are approximately 13 known closely related MTs expressed in the human body.

In humans, large quantities of these proteins are synthesized primarily in the liver and kidneys. However they have been found in other sites of the body as well. Their synthesis is dependent on the availability of the dietary minerals and toxic metals present in the body. This protein has properties like detoxification of toxic heavy metals, homeostasis of vital metals, oxidative stress, cell survival, protection against DNA damage, apoptosis, angiogenesis, as well as increased cell proliferation in the body (Higashimoto *et al*., 2009).

Figure 3

Schematic Representation of the Stimuli that Induce MT and the Downstream Effects of MT Over Expression



Takahashi (2012)

MT can be triggered by an array of stimuli including metal ions, oxidative stress, cytokines, growth factors and radiation. Downstream effects of MT over expression are free radical scavenging activity, tumor suppressor protein p53 and nuclear transcription factor NF-κB. All these downstream MT effects influence drug resistance, cell survival, cell growth and differentiation (Figure 3).

Stimulation of MT is particularly metal dependent. MT binds metals through the thiol (-SH) moieties of its cysteine residues. Usually, MT has high attraction to both essential metals namely Zn and Cu, and nonessential (or toxic) metals namely Cd, Ag and Hg which give rise to metal-thiolate clusters (Kagi and Vallee, 1960; Robbins *et al.*, 1991). The MT binding affinity to metal ions is different between various metals as discussed in several *in vitro* studies. It showed a different relative order of affinity to metals as Hg > Ag >> Cu > Cd > Zn (Hamer *et al*., 1986). Furthermore, MT has greatest stability for Hg followed by Ag, Cu, Cd and Zn. According to both the binding affinity and stability of MT indicate that metal ions namely Cd, Ag, Hg or Cu are more capable to displace Zn to further forming the more stable CdMT, AgMT, HgMT or CuMT complexes than other low affinity and stability metal ions (Sabolic *et al.*, 2010).

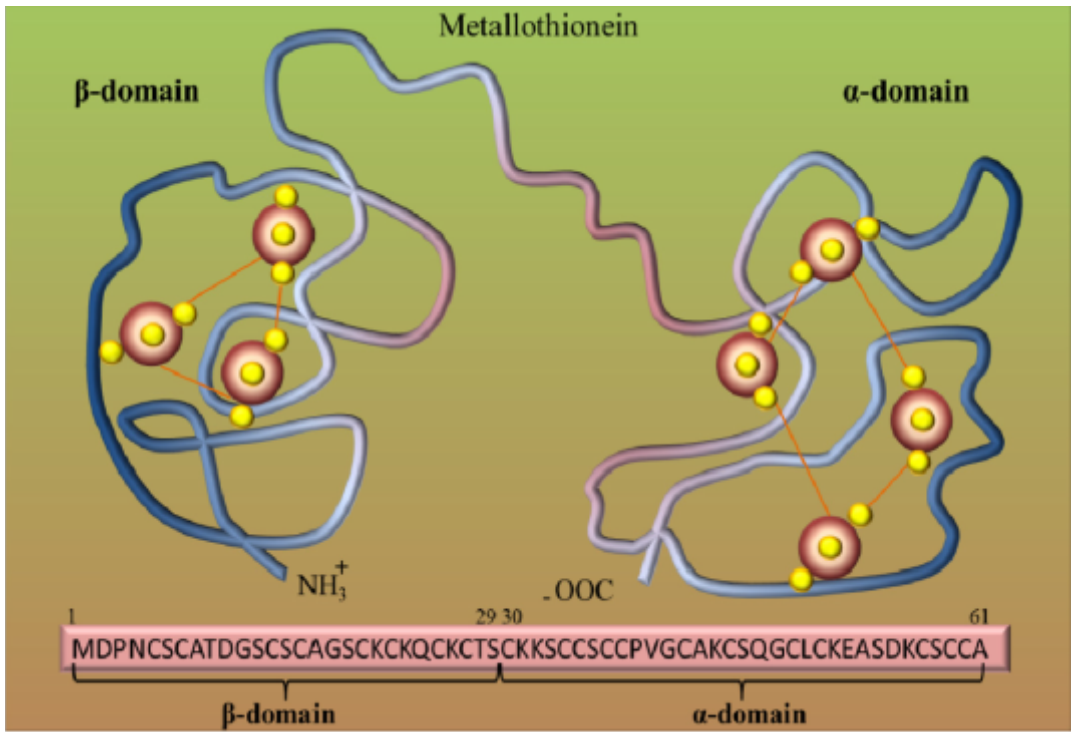
2.7.2 Structure of metallothioneins

Mammalian MTs may contain 61–68 amino acids and among them 20 are cysteines (Kagi and Schaffer, 1988; Romero-Isart and Vasak, 2002). This unique protein is involved in diverse intracellular functions (Davis and Cousins, 2000). However its role in detoxification of toxic heavy metals and in the maintenance of vital metal ion homeostasis is widely investigated, because this protein has high affinity towards metals (Klaassen *et al*. 2009; Templeton and Cherian, 1991).

Based on the structural models, it can be believed that MT is composed of two binding domains namely α and β which are composed of cysteine clusters. Sulfhydryl cysteine residues in MT are involved in covalent binding of metal atoms (Figure 4).

Figure 4

Structural Model of Two Binding Sites of Metallothionein



Red big beads are metal atoms and small yellow beads are sulfur atoms

Petrlova *et al*. (2006)

The *N*-terminal part of the peptide is designated as β-domain and has three and five binding sites for divalent and monovalent ions respectively. The C-terminal part of the peptide is designated as α-domain and has the potential to bind four and seven divalent and monovalent metal ions respectively (Ruttkay-Nedecky *et al*., 2013). Binz and Kagi (1999) indicated that MT can integrate as many as 7 divalent metal atoms (Zn^{2+}) or 12 monovalent atoms (Ag^{+}).

2.7.3 Metallothionein isoforms

In mammals MT gene family consists of four subfamilies designated as MT-1, MT-II, MT-III and MT-IV (Carpene *et al*., 2007). In humans, the MT genes are located on chromosome 16 in a cluster and consist of 16 identified genes in which five are pseudogenes. While the MT-II, MT-III and MT-IV proteins are encoded by a single gene,

MT-I protein consists of many subtypes encoded by a set of 13 MT-I genes (Moleirinho *et al*., 2011)

MT-I and II are present almost in all types of cells throughout the body. They regulate essential metal ions namely copper and zinc that are involved in cell transcription, play a role in immune function and detoxify heavy metals. MT-III is found primarily in the brain but present in heart, kidneys and reproductive organs also and it plays a major role in the development, organization and programmed death of brain cells. MT-IV is found in the skin and upper stomach. It regulates stomach acid pH, protects against sunburn and other skin traumas and helps to taste or texture discrimination of the tongue. (Moffatt and Seguin, 1998; Uchida *et al*., 1991; Quaife *et al*., 1994; Thirumoorthy *et al*., 2011)

2.7.4 Metallothioneins in oxidative stress

MT was mostly believed to be a protein that is involved in detoxification of overloaded essential and non essential metals. Its role is still argued by most researchers working in the MT field and often in agreement with data from fungi to mammals which could elucidate the broad diversity of MT isoforms.

Egli *et al*. (2006) studied on the role of MT in heavy metals homeostasis and detoxification in *Drosophila melanogaster*. It has four MT genes. They are transcriptionally provoked by heavy metals through the metal responsive transcription factor (MTF-1). Targeted mutagenesis showed that the four MT genes exhibited distinct and overlapping role in heavy metal detoxification and homeostasis. According to Gehrig *et al*. (2000), metal binding ability of metallothionein was confirmed by electrospray ionization mass spectrometry of zinc, cadmium, and copper. A copper-specific MT isoform was proved to preferentially bind 12 copper ions in the Roman snail, *Elix pomatia*.

Metallothioneins in marine molluscs was reviewed by Isani *et al*. (2000), who reported that cadmium, copper and mercury were considered as excellent inducers of MT biosynthesis and it could be used as a valid biomarker for metal exposure in marine molluscs.

Heat resistant and cysteine containing protein of 35 kDa was evidenced by Alhama *et al*. (2006) in digestive gland of clams from metal exposed southern Spanish coastal sites. They also proposed that MT could be used as a suitable biomarker for metal exposure monitoring programmes.

MTs could be induced by essential metals namely Cu and Zn and non essential metals namely Cd, Ag and Hg in both vertebrates and invertebrates. MTs also were found to play an important role in routine metabolic handling of essential metals and also in detoxification of excess amounts of essential and non essential metals as reported by Amiard *et al*. (2006).

With regard to terrestrial animals, a significant association between MT and cadmium levels in kidney was illustrated in wild animals including earthworm, *Allolobophora caliginosa*, Eurasian woodcock, *Scolopax rusticola* and wild mice, *Apodemus sylvaticus* highlighting the major role of MT in metal detoxification processes as pointed by Carpene *et al*. (2006) and Rogival *et al*. (2007).

2.8 Free Radical Scavenging Role of Metallothioneins in Oxidative Stress

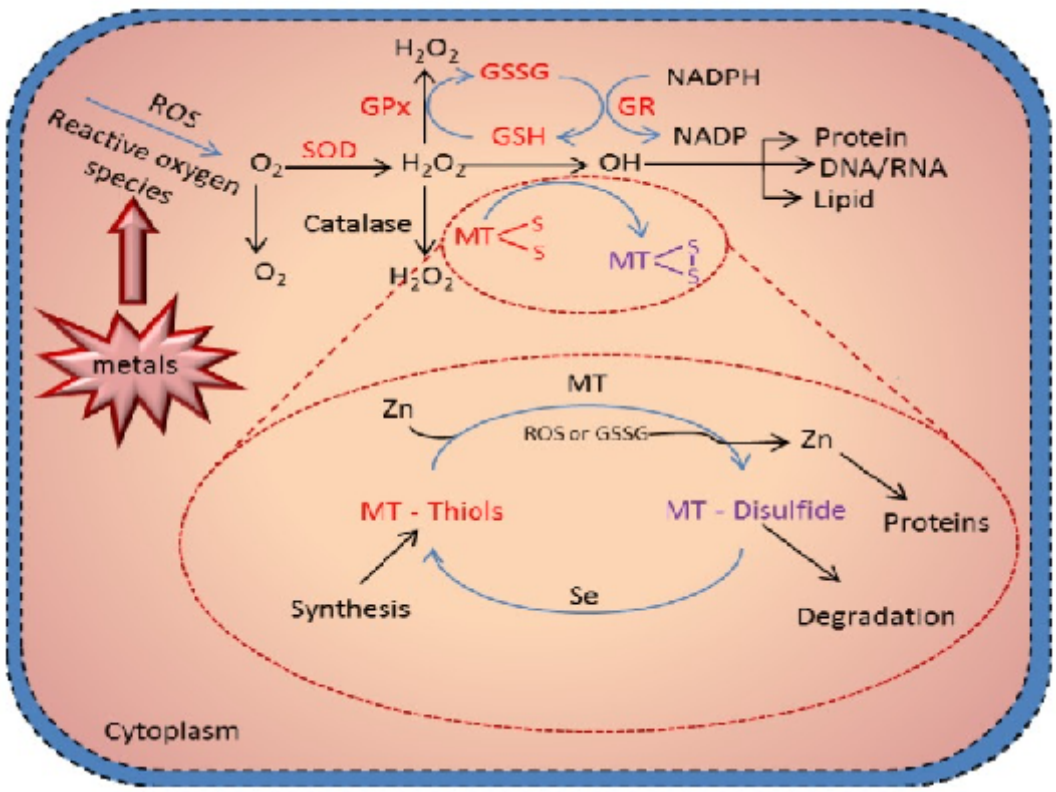
Free radicals are chemical particles containing one or more unpaired electrons, which may be part of the molecule. They cause the molecule to become highly reactive (Halliwell and Gutteridge, 1986).

Radicals and other reactive species are formed constantly in human body and are removed by the enzymic and non - enzymic antioxidant defense systems (Rakesh *et al*., 2010). Overproduction of reactive oxygen species (ROS) results in oxidative stress and is a deleterious process that can be an important mediator of damage to cell structures, including lipids, proteins and DNA. Antioxidants are compounds able to either delay or inhibit the oxidation processes which occur under the influence of ROS (Pisoschi and Negulescu, 2011). Various studies have shown that metals are capable of producing the oxygen free radicals. Toxicities of metal ions may be related to differences in solubility, absorbability, transport and chemical reactivity that are formed within the body. Antioxidants exert synergistic actions in scavenging free radicals.

A study on administration of $CdCl_2$ and $AgNO_3$ in mice showed that both the salts enhanced the synthesis of MTs and also served as an effective scavenger of reactive oxygen species as demonstrated by Srivastava *et al*. (1995).

Figure 5

Scavenging of Reactive Oxygen Species in Metallothioneins Redox Cycle



Kang (2006)

MT is known to react with reactive species and radicals and has been considered as an antioxidant. MT has specific redox properties that selectively control release and uptake of essential metals randomly and infrequently. There is growing evidence that MT possesses the ability to scavenge an array of radicals including hydroxyl ($OH^{\bullet}$), super-oxide ($O_2^{\bullet}$) and organic radicals. The radical scavenging potential of MT has been studied by *in vivo*, cell cultures and cell free experiments (Kiningham and Kasarskis, 1998).

In the presence of redox metals namely Cu and Fe, a cell can produce ROS which can lead to damaging of DNA and cell structures. The cell protects itself using various molecules as scavengers of the radicals. One of the most significant cell pathways to scavenge the radicals is the glutathione redox complex. But, free –SH moieties of MT can be also involved in the scavenging of ROS in the MT redox cycle (Figure 5) (Ruttkay-Nedecky *et al*., 2013).

Under physiologic conditions, zinc bound to MT is released through oxidation of the thiolate cluster when the environment becomes oxidized. Formation of MT-disulfide would be subjected to degradation. MT disulfide is reduced to MT- thiol. This reduction process is greatly enhanced in the presence of selenium catalyst. In presence of zinc, MT is quickly reconstituted. This process constitutes the MT redox cycle which plays a crucial role in the biological function of MT (Ruttkay-Nedecky *et al*., 2013).

Several *in vitro* studies had proved that MT functions as an antioxidant against reactive oxygen and nitrogen species. Studies using a cell-free system have demonstrated the ability of MT as a free radical scavenger (Abel and Deruiter 1989; Thornalley and Vasak 1985; Cai *et al*. 2000). Metallothionein has been shown to scavenge hydroxyl radicals *in vitro*, because of its cysteinyl thiolate groups (Sato and Bremner, 1993).

The mechanism by which MT scavenges oxygen free radicals was elucidated in cell-free systems by Thornalley and Vasak in 1985. They revealed that rabbit liver metallothionein capable to scavenge free hydroxyl ($OH^{\bullet}$) and superoxide ($O_2^{\bullet}$) radicals produced by the xanthine/xanthine oxidase reaction and all 20 cysteine sulfur atoms were involved in the radical quenching process.

Coppen *et al*. (1988) reported that Zn inhibits free radical production and lipid peroxidation in cultured hepatocytes subjected to oxidative challenge. They revealed that the mode of action of Zn could occur via free radical scavenging by zinc- induced metallothionein.

Romero-Isart and Vasak (2002) used an epithelioma cell line from a piscine species, *Cyprinus carpio* and demonstrated protective effect of MT in free radical scavenging when cells were treated with cadmium.

An *in vitro* experiment was conducted by Davis and Cousins (2000) to know the mechanisms of metallothioneins with cadmium which showed that S-nitrosothiols are formed in beta domain of mouse Cd7MT1 with a subsequent random formation of disulfide bonds. This result revealed that MT was involved in detoxification of cadmium.

Andrews (2000), reported that MT of higher eukaryotes were involved in regulating zinc levels and distribution within cells and organisms. This protein could also defend against toxic metals and oxidative stress inducing agents. In mice, among the four identified MT genes, MT-I and -II genes were most widely expressed. Transcription of these genes was quickly up regulated in response to heavy metals as well as oxidative stress and inflammation causing agents. The six zinc finger metal responsive transcription factor MTF-1 played an essential role in transcriptional activation of the MT-I gene in response to metals and oxidative stress. Regulation of the mouse MT-I gene by metals and oxidative stress agents seemed to be involved in multiple signaling pathways that were dependent on the species of metal ion and the nature of the oxidative stress agents.

2.9 Metallothioneins as a Biomarker of Silver Exposure

Several previous data from *in vivo and in vitro* studies have suggested that silver nanoparticles induce toxicity to living beings. According to Choi *et al*. (2010), Zebrafish was exposed to an Ag-NP solution in which free Ag^+ ions was not present at the time of treatment. However, the metal sensitive metallothionein 2 (MT2) mRNA was induced in the liver tissues of Ag-NP treated Zebrafish, suggesting that Ag^+ ions was released from Ag-NP after treatment. They also opined that there was a possibility that MT2 mRNA were induced in the liver tissues by Ag-NP generated free radicals. A number of cellular alterations including disruption of hepatic cell cords and apoptotic changes were observed in histological analysis of the liver tissues.

Luther *et al*. (2012) tested the prolonged consequences of a short transient exposure to silver nanoparticles in cultured astrocytes. Acute exposure of astrocytes to Ag-NP led to a concentration dependent increase in the specific cellular silver content up to 46 nmol/mg of protein. But they did not observe any change in cell viability. Western blot analysis and immunocytochemical staining revealed that Ag-NP treated astrocytes strongly upregulated the expression of MTs.

A toxicity study of silver nitrate ($AgNO_3$), silver chloride ($AgCl_n$) and silver thiosulfate [Ag $(S_2O_3)_n$] with juvenile rainbow trout, *Oncorhyncus mykiss* was performed by Hogstrand *et al*. (1996). Exposure to $AgNO_3$, Ag $(S_2O_3)_n$ and $AgCl_n$ led to accumulation of Ag and induction of MT. Highest Ag level was observed in liver of trout exposed to 164,000 µg Ag L^{-1} as $Ag(S_2O_3)_n$. The hepatic Ag level was increased 335 times in exposed fish from control value. The MT levels were also increased in gills and liver and highest level of MT was found in liver of fish exposed to $Ag(S_2O_3)_n$.

Kaewamatawong *et al*. (2014) studied that acute and sub-acute pulmonary toxicity of colloidal silver nanoparticles (0 ppm - 100 ppm) of Ag-NPs were instilled intratracheally in mice. Their findings suggested that instillation of Ag-NPs caused transient moderate acute lung inflammation and tissue damage. Oxidative stress might trigger the induction of injury to lung tissue. Additionally, expression of MT in tissues indicated the protective response to exposure to Ag-NPs.

Based on the above facts and previous studies, an attempt was made to understand the expression of metallothioneins in the presence of silver compounds through *in vivo* and *in vitro* system.