

Induction of Metallothionein in Rat Liver by Zinc Exposure: A Dose and Time Dependent Study

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Abstract Metallothioneins (MTs) are low molecular weight ubiquitous metalloproteins with high cysteine (thiol) content. The intracellular concentration of zinc (Zn) is tightly regulated and MT plays a crucial role in it. The present study investigates the relationship between the Zn status (as a function of Zn concentration and time) in the rat liver and the occurrence of hepatic MT. For dose dependent study, four experimental groups, one control and three receiving different levels of metal supplementation, were chosen [Group 1 control and Group 2, Group 3, Group 4 receiving subcutaneous dose of 10, 50 and 100 mg of Zn/kg body weight (in the form of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), respectively]. For the time dependent expression of MT, again four experimental groups, i.e. Group 5 control and Group 6, Group 7, Group 8 receiving 50 mg of Zn/kg body weight (in the form of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) subcutaneously and sacrificed at different time intervals after last injection i.e. 6, 18, 48 h, respectively were chosen. Isolation of MT was done by using combination of gel filtration and ion exchange chromatography while characterization of MT fraction was carried in the wavelength range 200–400 nm. Expression of MT was studied by using Western blot analysis. The results revealed that the MT expression increases with increasing the dose of Zn administered and maximum at 18 h after last Zn injection. Accumulation of MT with increase dose would help in maintaining the intracellular Zn concentration by its sequestration which further reduces the possibility of undesirable binding of Zn to other proteins significantly and maintains

Zn homeostasis. The maximum expression of MT at 18 h is indicative of its half life.

Keywords Metallothionein · Zinc · Chromatography · UV–vis spectra · Western blot analysis

Abbreviations

MT	Metallothionein
LD	Lethal dose
MTF-1	Metal transcription factor-1
MRE	Metal responsive element
DEAE	Diethylaminoethyl
BSA	Bovine serum albumin
MWCO	Molecular weight cut-off
A_{280}	Absorbance at 280 nm
A_{205}	Absorbance at 205 nm
ESI-MS	Electrospray ionization mass spectrometry
LMCT	Ligand to metal charge transfer

1 Introduction

Zinc (Zn) is one of the most copious essential transition metal, lacking biological redox activity [1, 2]. In the biological systems, various roles are performed by Zn e.g. the catalytic (as in carboxypeptidase A and as in alkaline phosphatase), the structural (as in alcohol dehydrogenase, zinc finger) and the regulatory (as in transcription factor IIIA) [3–6]. Zn is involved in various physiological processes which are essential for cellular growth, proliferation and apoptosis [7–11]. The liver is an important organ for the storage and homeostasis of Zn [12].

Tightly controlling the intracellular concentration of Zn i.e. pico to nanomolar levels is emerged as one of the most important mechanism employed for regulating different

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cellular processes [13]. The tight control on the intracellular Zn ions concentration is also evident from the fact that Zn salts have very high LD₅₀ values in different mammalian species e.g. the intraperitoneal injection LD₅₀ is 28–73 mg/kg whereas the oral LD₅₀ is 237–623 mg/kg for zinc salts [14, 15]. Also, for zinc chloride, the inhalation LD₅₀ is 2000 mg/m³ [16]. The humans may receive such high acute doses of Zn, only under some unusual circumstances. Zn transporters (e.g. Slc39/ZIP and Slc30/ZnT families) and Zn-sensing molecules such as metallothionein (MT) are responsible for the tight control of low cellular Zn levels [17–22].

MTs are low molecular weight (6–7 kDa) metal binding proteins with high thiol content. They are the only cellular Zn storage proteins yet identified. They regulate the translocation of Zn ions from one cellular compartment to another [23]. Firstly, the binding of intracellular free Zn²⁺ ions to the zinc-fingers of the Metal Transcription Factor-1 (MTF-1) protein takes place. As a result of this, the MTF-1 gets translocated from the cytosol to the nucleus where it binds to the metal-responsive elements (MRE) present in the promoter region of MT gene and initiates MT expression [24]. The oxidation and reduction state of MT is governed by the release and binding of Zn to it. Because of this reason, it can serve as both zinc acceptor (under more reducing conditions) and zinc donor (under more oxidizing conditions) [13, 25, 26]. Therefore, it can be concluded that cellular accumulation of MT plays a crucial role in zinc metabolism and maintaining its low intracellular concentrations. The cellular accumulation of MT can be achieved by altering its gene expression and degradation pattern. The present study is designed to determine the mechanism for the cellular accumulation of MT in condition of excess Zn administration i.e. whether it is attained by increasing the MT expression at translational level or by altering its degradation pattern or by both processes. To achieve this, a dose and time dependent MT expression was studied in the rat liver. Isolation of MT was done by using the combination of Gel filtration and ion exchange chromatography and the characterization of MT fraction was done using UV–visible spectroscopy in the wavelength range 200–400 nm. MT protein expression was studied using Western immunoblot analysis.

2 Materials and Methods

2.1 Chemicals

Sephadex G-75 and DEAE-Sephadex A-50 were obtained from Sigma Aldrich Co., USA Zinc sulphate, sodium chloride, sodium hydroxide, trisacetate, sodium carbonate, copper sulphate, sodium potassium tartrate, Folin's reagent, bovine serum albumin (BSA), lysozyme, glutathione

were procured from Central Drug House (P) Ltd, New Delhi, India and of analytical grade.

2.2 Animal Treatment

Male Wistar rats having the body weight in the range 150–250 g were attained from the Central Animal House facility available at Panjab University, Chandigarh (India). For dose dependent study, the rats were randomly divided in four groups (eight animals each) i.e. Group 1, Group 2, Group 3, Group 4. Animals in Group 1 (control) were injected subcutaneously (sc) with normal saline (0.9%), once a day for 2 days. Animals in Group 2, Group 3, and Group 4 were injected sc with 10, 50 and 100 mg of Zn/kg body weight (in the form of ZnSO₄·7H₂O), respectively, once a day for 2 days. Different doses of Zn were prepared in 0.9% of saline. After 18 h of the last injection the animals were sacrificed by cervical dislocation under anesthesia and the livers were excised. For time dependent study, the rats were again randomly divided in four groups (eight animals each) i.e. Group 5, Group 6, Group 7, and Group 8. Animals in Group 5 (control) were injected subcutaneously (sc) with normal saline (0.9%), once a day for 2 days and immediately sacrificed after the second injection. The animals in Group 6, Group 7, and Group 8 were injected sc with 50 mg of Zn/kg body weight (in the form of ZnSO₄·7H₂O), once a day for 2 days. After the last injection, the animals were sacrificed at different time points i.e. Group 6 after 6 h, Group 7 after 18 h and Group 8 after 48 h. All experimental procedures involving the rats were preapproved by the Ethical Committee on animal experiments, Panjab University, Chandigarh.

2.3 MT Isolation

MT protein isolation from the rat liver of control and different treatment groups was performed by the procedure of Bremner and Davies [27] using combination of gel filtration and ion exchange chromatography with slight modifications. For the selection of chromatographic fractions corresponding to MT, Bremner and Davies [27] had used the presence of zinc as the criteria. However, in the present study, the presence of absorbance at 205 nm (A₂₀₅) and absence of absorbance at 280 nm (A₂₈₀) were also chosen as selection criteria in addition to zinc. As the mammalian MT lacks aromatic amino acids, they lack A₂₈₀ and only show absorbance due to peptide bonds at 205 nm (A₂₀₅) [28]. The detailed protocol is reported earlier [29]. For the purity and identification of the isolated MT, standard procedure of Laemmli was used [30] on minigel apparatus (Biorad, UK) and the staining of the final gel was done using silver stain [31].

2.4 UV Spectral Analysis

Absorption spectra of isolated MT were measured by using UV–visible spectrophotometer (Shimadzu Model UV 1800).

2.5 MT Expression Analysis

The MT1 expression at translational level was studied using the Western immunoblot analysis. The detailed procedure is reported earlier [32]. This method was also used earlier for the analysis of MT protein expression [33, 34].

2.6 Statistical Analysis

One-way analysis of variance (ANOVA) was used for the determination of statistical significant difference among different groups, followed by multiple post hoc least significant difference (LSD) test for calculating the comparisons between different treatment groups. For the processing of the

data, the statistical analysis software SPSS (14 version) was employed. Data are expressed as mean \pm standard deviation with $p \leq 0.05$ as the level of significance in all cases.

3 Results

3.1 Standardization of MT Isolation Procedure

Protocol for MT isolation was first standardized for Group II (i.e. 10 mg of Zn/kg body weight) in our lab and later the same procedure was performed for all others groups. Figure 1a, b show the protein (A_{280} , A_{205}) and Zn (ppm) analysis in gel filtration and ion exchange fractions of the rat liver after Zn supplementation, respectively.

On the basis of absorbance observed for the gel filtration fractions, they could be divided into three regions. F1 (10–22) shows significant absorbance at A_{280} , A_{205} and Zn (ppm). The fractions obtained are heterogeneous and may

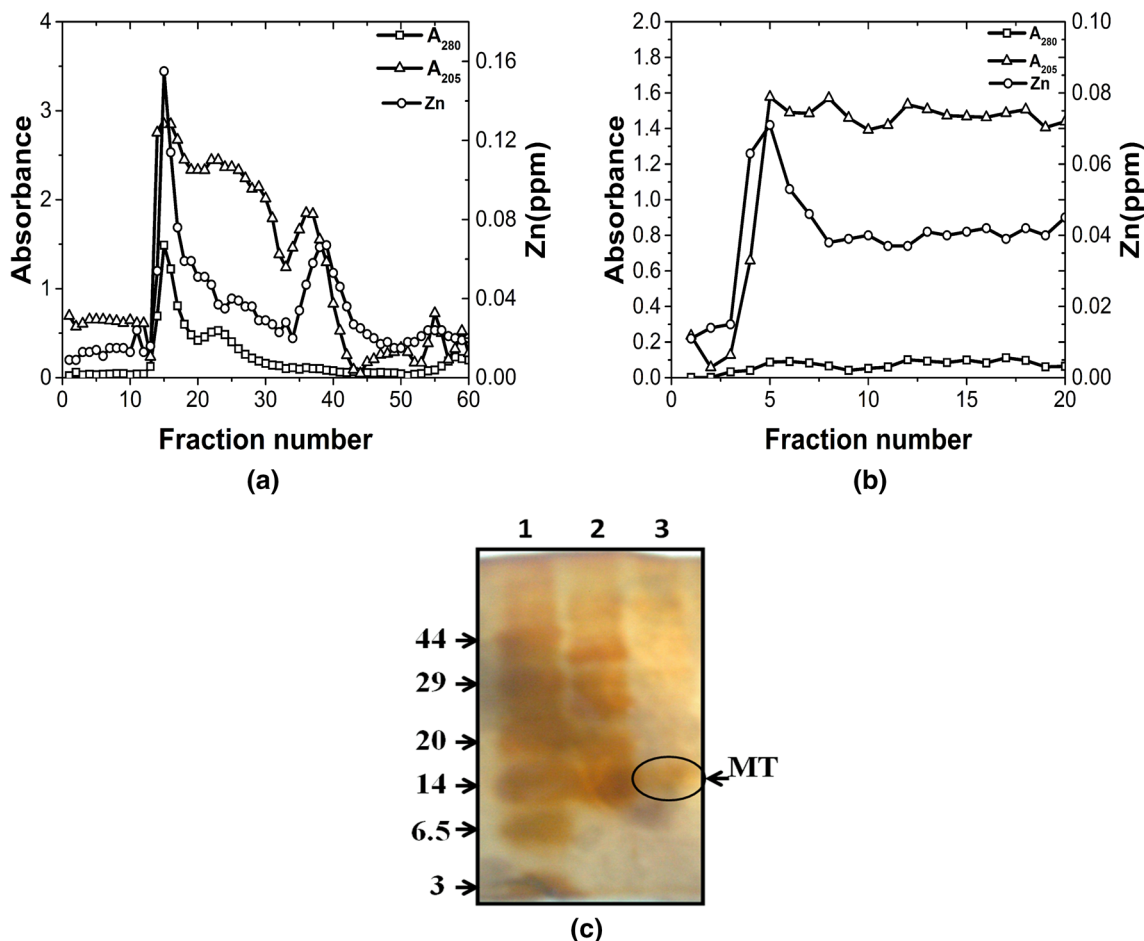


Fig. 1 Protein (A_{280} , A_{205}) and Zn (ppm) analysis in **a** gel filtration fractions and **b** ion exchange fractions of liver cytosol from Group 2 rats (10 mg/kg body weight of Zn). **c** MT analysis by SDS-PAGE and silver staining. Lanes: (1) molecular weight markers of different

molecular weights i.e. 3, 6.5, 14, 20, 29 and 44 kDa (2) hepatic cytosolic fraction (3) isolated MT fraction. Lane 3 shows single band at 14 kDa corresponding to MT

contain high molecular weight Zn binding proteins like superoxide dismutase, carbonic anhydrase, glycerol-3-phosphate dehydrogenase and Ovalbumin [28]. The second region F2 (24–44) shows considerable Zn content, significant A_{205} , whereas A_{280} was negligible for these fractions. Mammalian MT was eluted in these fractions [32]. So, these fractions were pooled for further processing. Third region (F3) consist of remaining fractions which showed little absorbance at both wavelengths. After multiple column runs, it was observed that the MT containing fraction (rich in Zn content, significant A_{205} , and negligible A_{280}) comes approximately between 6 and 11 h or 24th–44th fractions column run. The pooled F2 fractions were further applied for anion exchange chromatography.

The pooled F2 fractions were further fractionated on DEAE Sephadex A-50 anion exchange chromatography. Eluted fractions from ion exchange for group II were analyzed for A_{205} , A_{280} and Zn (ppm). One sharp peak F-1* (fractions 2–9) and flat curve F-2* (fractions 10–20) of zinc were seen among the anion exchange fractions. The fractions F-1*(2–9) were then pooled and concentrated using Centricons® (Millipore, 3 kDa MWCO). From the silver stained gel, it was found that multiple bands were present in liver cytosol sample (Fig. 1c, lane 2). However, in the isolated protein sample, a single band at 14 kDa was observed (Fig. 1c, lane 3). The observed band at 14 kDa [twice the size of monomeric MT (6–7 kDa)], is due to intermolecular metallation in the isolated MT [35].

In the present study, the characteristic of the chromatographic fractions corresponding to MT showing higher A_{205} and negligible A_{280} is efficiently exploited for the isolation of MT from the liver of rats of different treatment groups. We were able to isolate pure protein as depicted by a single band corresponding to isolated MT

samples in the SDS-PAGE with silver staining (Fig. 1c). The Electrospray ionization mass spectrometry (ESI-MS) was used for the confirmation of molecular weight of isolated MT [29].

3.2 Gel Filtration

The graphs for A_{280} and A_{205} analysis in the MT fractions for Group 2, Group 3 and Group 4 (which varies in the amount of Zn supplementation) are shown in left panel of Fig. 3. The pattern of graph for A_{280} and A_{205} was very similar in all treated groups. In control group (Fig. 2a), the A_{205} was very low as compared to the rest of the groups. For the F 2 fractions, the average A_{205} was calculated for all the groups. The average A_{205} for Group 2, Group 3 and Group 4 is 1.19 ± 0.77 , 1.32 ± 0.54 and 1.38 ± 0.32 , respectively. This implies that with the increase in the doses of Zn received by the animals, the average A_{205} increases in the gel filtration fractions corresponding to MT i.e. maximum for Group 4 animals receiving 100 mg/kg body weight of Zn, then Group 3 animals receiving 50 mg/kg body weight of Zn and minimum for Group 2 animals receiving 10 mg/kg body weight of Zn.

The graphs for A_{280} and A_{205} analysis in the MT fractions for Group 6 and Group 8 are shown in left panel of Fig. 4. As the Group 3 and Group 7 received similar metal treatment, the results are shown only once in Fig. 3. Similar trends for A_{280} and A_{205} were observed for the gel filtration fractions of liver cytosol from Group 6 and Group 8 rats to that of groups 2, 3 and 4 (Figs. 3, 4, left panel). Group 6 have average $A_{205} = 1.04 \pm 0.41$, while Group 8 have average $A_{205} = 1.20 \pm 0.80$. The maximum average A_{205} in the gel filtration fractions corresponding to MT was observed

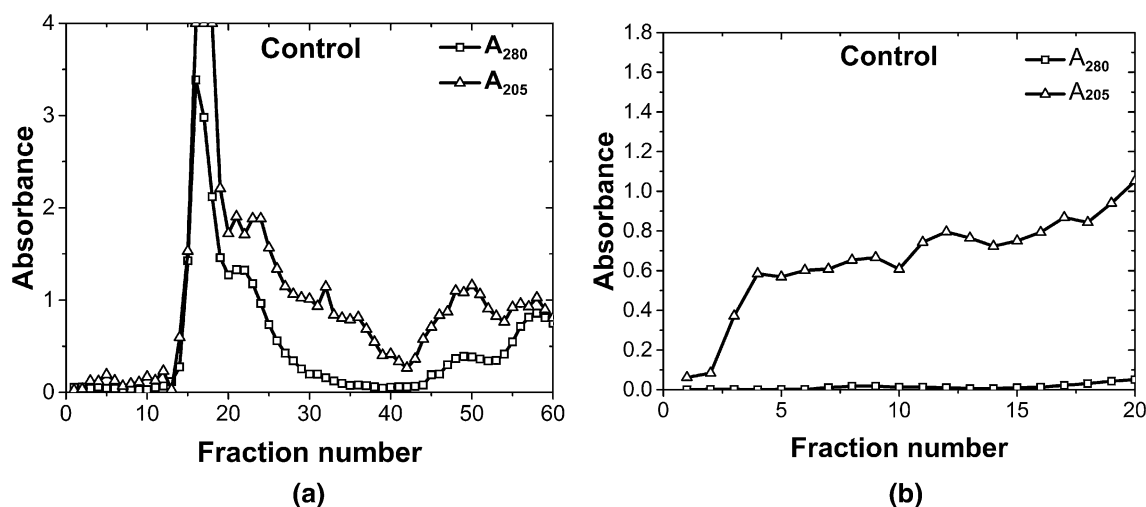


Fig. 2 Protein (A_{280} , A_{205}) analysis in **a** gel filtration and **b** ion exchange fractions of liver cytosol from animals of control group

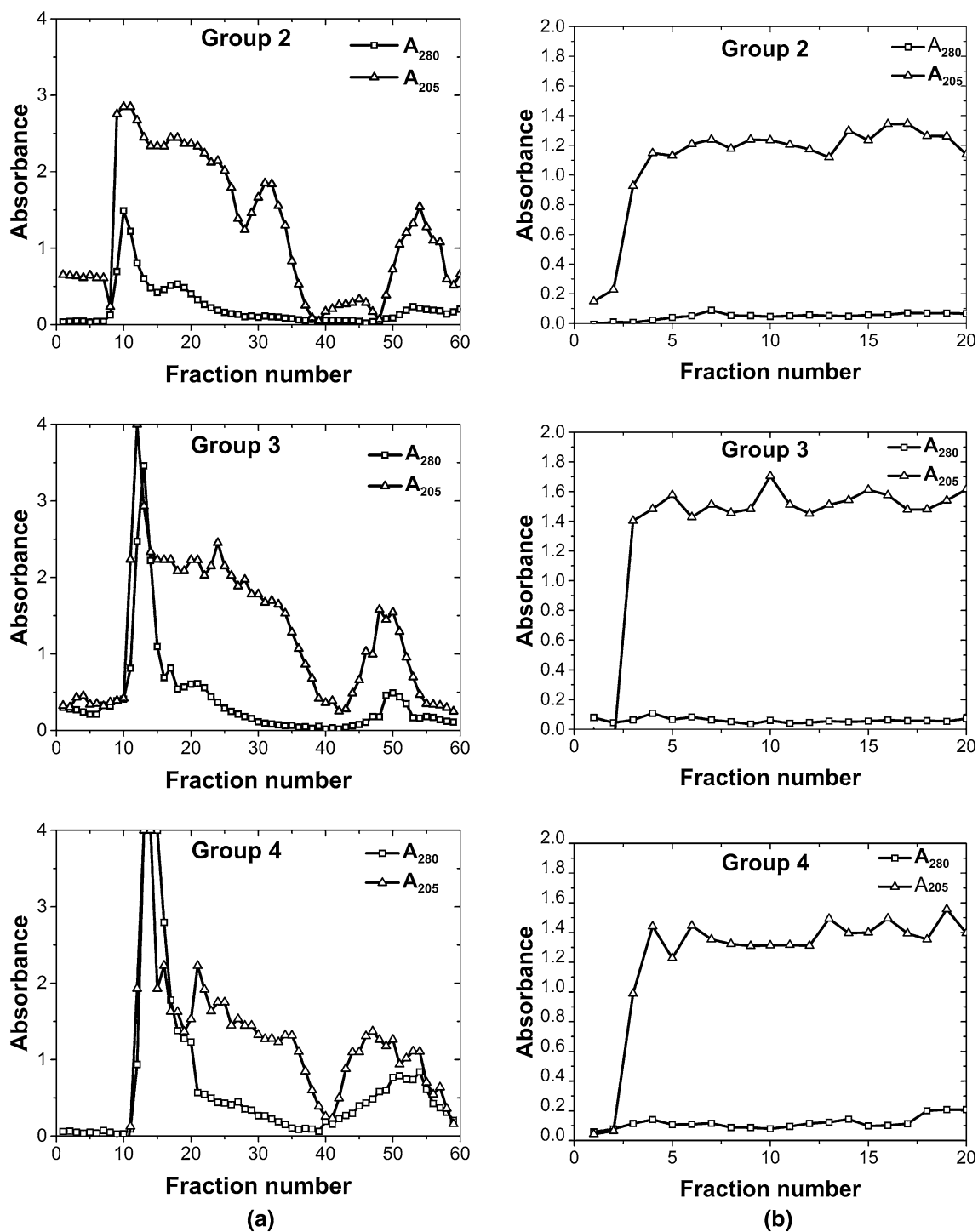


Fig. 3 Protein (A_{280} , A_{205}) analysis in **a** gel filtration and **b** ion exchange fractions of liver cytosol from Group 2, Group 3 and Group 4 rats

for Group 7, then Group 8 and the minimum for Group 6 animals dissected 18, 48 and 6 h after the second injection of the Zn, respectively.

3.3 Ion Exchange

After anion exchange chromatography, again the pattern of graph for protein (i.e. A_{280} , A_{205}) was similar in Group 2, Group 3 and Group 4, although the magnitude varies (Fig. 3, right panel). For the F-1* (2–9) fractions

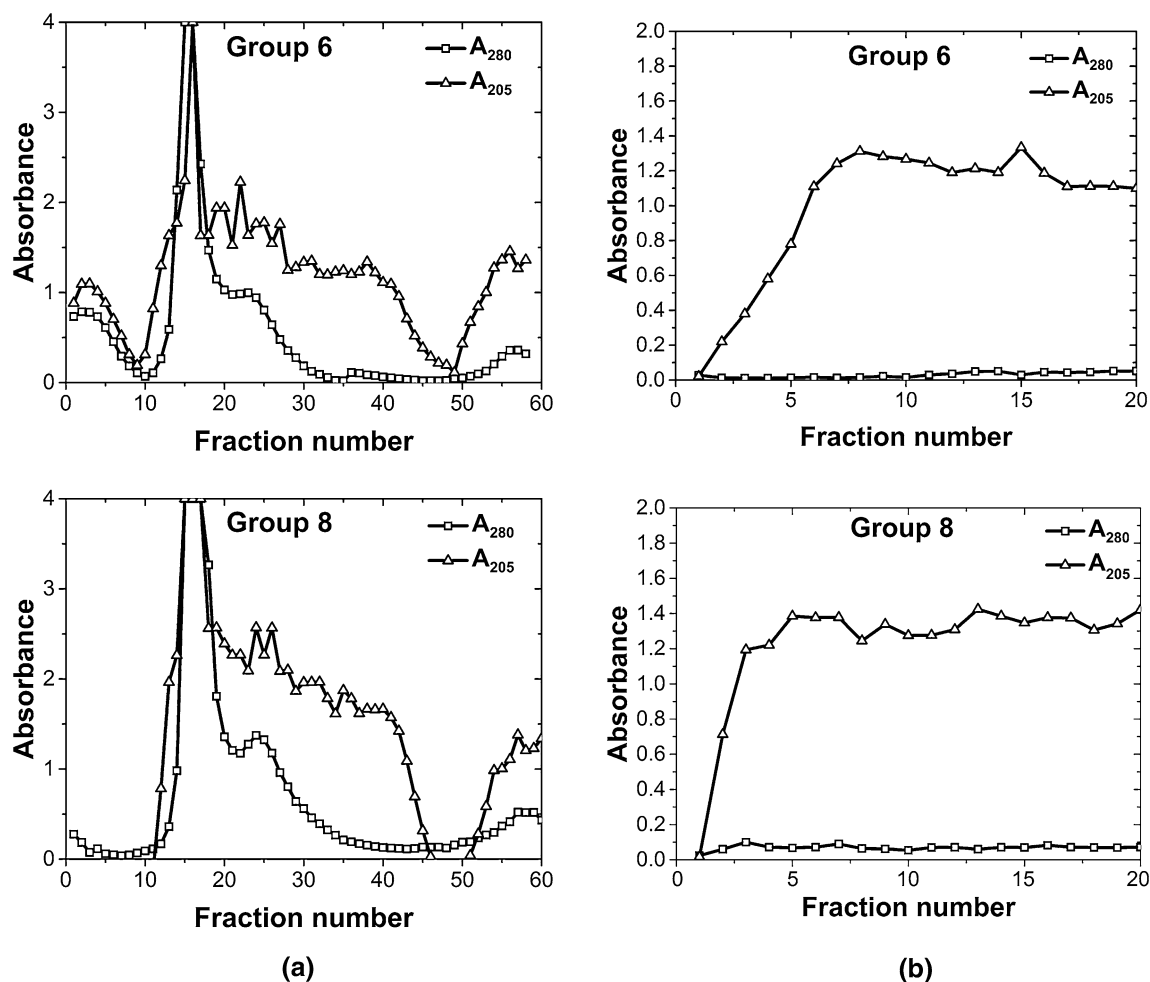


Fig. 4 Protein (A_{280} , A_{205}) analysis in **a** gel filtration and **b** ion exchange fractions of liver cytosol from Group 7 and Group 8 rats

of ion exchange, the mean A_{205} was calculated for all groups. The mean A_{205} for Group 2, Group 3 and Group 4 is 1.08 ± 0.29 , 1.35 ± 0.49 and 1.37 ± 0.22 , respectively. Again, with the increase in the doses of Zn received by the animals, the average A_{205} increases in the ion exchange fractions corresponding to MT.

The graphs for A_{280} and A_{205} analysis in the ion exchange fractions for Group 6 and Group 8 are shown in right panel of Fig. 4. Group 6 have average $A_{205} = 1.04 \pm 0.41$, while Group 8 have average $A_{205} = 1.20 \pm 0.80$ for F-1* fractions of ion exchange chromatography. The maximum average A_{205} in the ion exchange fractions corresponding to MT was observed for Group 7, followed by Group 8 and minimum for Group 6 animals dissected 18, 48 and 6 h after the second injection of the Zn, respectively, similar to gel filtration fractions.

Fractions (2–9) were pooled from all treatment groups and concentrated with the help of Centricons[®], Millipore, MWCO 3 kDa.

3.4 UV–Vis Spectral Analysis of Isolated MT

The absorption bands of MTs are usually observed in the ultraviolet wavelength range (between 200 and 400 nm) because of the phenomenon of ligand to metal transfer (LMCT) [36]. The absorbance of aromatic groups and disulphide linkages would normally mask this region, which are absent in MT. Hence, this technique is routinely used for observing the metallation process in MTs [37–41]. The UV–visible spectra of the MT samples isolated from different treatment groups contain two peaks, first peak in the wavelength range 205–225 nm and second peak in the wavelength range 270–275. For different treatment groups, the λ_{\max} and corresponding absorbance was calculated from the Fig. 5. Out of Group 2, 3 and 4 (which varies in doses of Zn received), the maximum absorbance was observed for the sample of isolated MT from the liver of Group 4 rats, then Group 3 and minimum was observed for the sample from Group 2 rats for both peaks. Out of Group 6, 7 and 8 (which

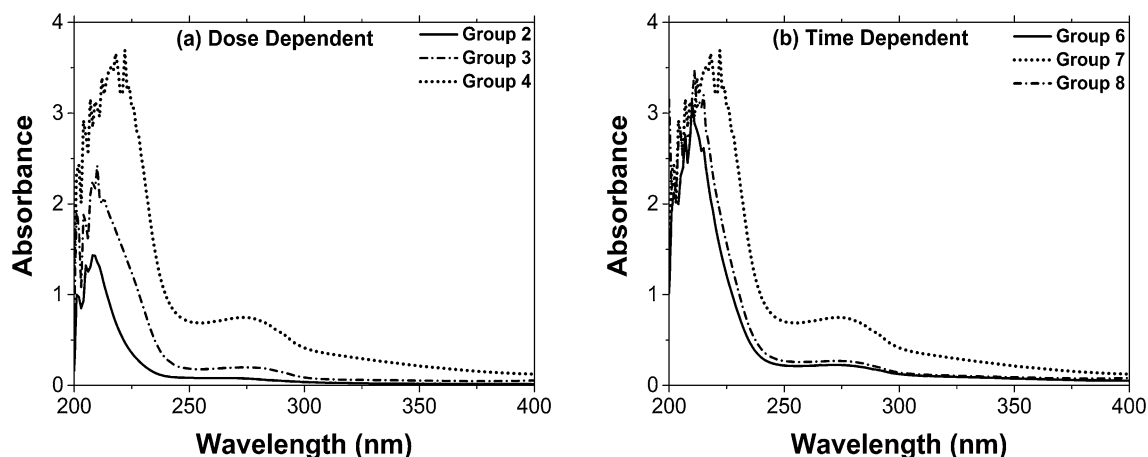


Fig. 5 Absorption spectra in UV region (i.e. between 200 and 400 nm) of the rat liver MT isolated from different supplemented groups: **a** dose dependent groups (i.e. Group 2, Group 3 and Group 4), **b** time dependent groups (i.e. Group 6, Group 7 and Group 8)

varies in the time of sacrifice of rats after the second injection of the Zn), the maximum absorbance was observed for the sample of isolated MT from the liver of Group 7 animals, then Group 8 and minimum was observed for the sample from Group 6 animals.

First peak (205–225 nm) arises due to absorbance of peptide bonds, while second peak (270–275 nm) [42] could be arisen due to presence of either aromatic amino acids or disulfide bonds. As aromatic amino acids are absent in mammalian MTs [28], the formation of disulfide bonds could be the reason for observing the second peak. Under oxidative conditions, the number of disulfide bonds present in MT increases due to release of zinc [43]. Moreover, the formation of intermolecular disulfide bonds is favored over intramolecular bonds because of the high concentrations of MT in vitro [35]. Also, MT1 was found to be more susceptible to air-oxidation [44].

3.5 Western blot analysis

MT expression at transcriptional level was performed using western blot analysis. For these experiments, liver PMF and cytosolic fractions of different treatment groups were used. The band corresponding to MT was observed at 14 kDa, similar to SDS-PAGE (Fig. 1c).

A significant increase in MT expression was observed for all treatment groups with respect to control. From the bar graphs (Fig. 6A(a)), it is observed that there is an elevation in MT levels in both PMF (Fig. 6A(b)) and cytosolic (Fig. 6A(c)) samples with increase in Zn doses (i.e. 10, 50 and 100 mg of Zn/kg body weight). To check the cross reactivity of antibody used, lysozyme and glutathione were loaded in lane 2 and 3, and no bands were observed in these lanes (Fig. 6A(a), B(a)). Thus, it was confirmed that the primary antibody used in the analysis was specific to MT.

A significant increase in MT expression was also observed in the PMF (Fig. 6B(b)) and cytosolic (Fig. 6B(b)) fractions prepared from for Group 6, 7 and 8 animals with respect to control (Group 5) animals. Western blot analysis revealed that maximum MT expression was observed for Group 7, followed by Group 8 and Group 6.

4 Discussion

The distribution of essential metals in the cellular compartments is performed by specialised proteins. There are different classes of these proteins on the basis of their functions: metal transporters (help in metal ions transportation through plasma membranes and different cellular compartments); metal-storage proteins; and metal-sensor proteins (sense the optimum concentration of specific metal inside the cells) [13]. Intracellular Zn is very important for the activities of many cellular enzymes and proteins, and its excessive accumulation is cytotoxic. Therefore, an assembly of cellular proteins is present to tightly control the cellular levels of Zn. These include zinc transporters as well as zinc binding protein metallothionein. In this study, we have investigated the time and dose dependent behaviour of MT expression in the rat liver on Zn supplemented higher than its hepatoprotective levels (above 100 μmol of Zn/kg sc [45]).

The average A_{205} for the chromatographic fractions corresponding to MT, peak intensities observed in UV spectra and western blots analysis revealed that out of Group 2, 3 and 4 (which varies in doses of Zn received), the MT expression is maximum in Group 4, minimum in Group 2 and Group 3 lies in between whereas, out of Group 6, 7 and 8 (which varies in time of dissection after the second injection of the Zn), the MT expression is maximum in Group 7 followed by Group 8 and Group 6 animals dissected 18, 48 and 6 h after

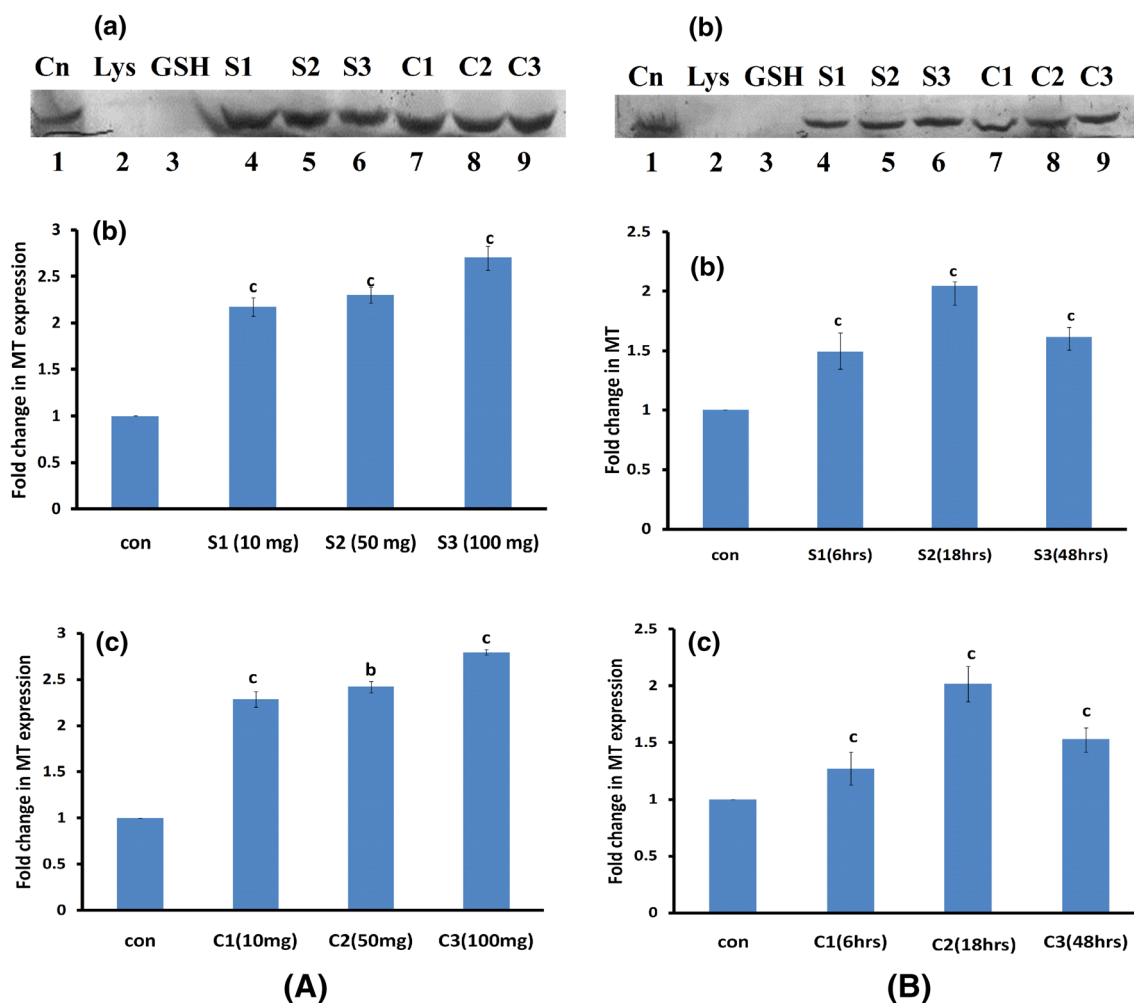


Fig. 6 a Western immunoblot analysis of MT (70 µg of protein) was carried in the PMF and cytosolic fractions. Lane 1–3 represents was control (Cn), lysozyme (lys), glutathione (GSH) in both **A(a)** and **B(a)**. Lane 4–6 represents the PMF for Group 2, Group 3 and Group 4, respectively in **A(a)** and Group 6, Group 7 and Group 8, respectively in **B(a)**. Lane 7–9 represents the cytosolic fractions for Group 2, Group 3 and Group 4, respectively in **A(a)** and Group 6, Group 7 and Group 8, respectively in **B(a)**. **b** Bar graph represents

the relative density values of 14 kDa band of MT in PMF samples. **c** Bar graph represents the relative density values of 14 kDa band of MT in cytosolic samples [the relative densities are expressed as average ± S.D. and is analyzed by one-way ANOVA followed by post hoc test ($b \leq 0.01$ significant w.r.t. control group. $c \leq 0.001$ significant w.r.t. control group) for **A** dose dependent groups (i.e. Group 2, Group 3 and Group 4), **B** time dependent groups (i.e. Group 6, Group 7 and Group 8)]

the second injection of the Zn, respectively. These results suggest that with the increase in the doses of Zn received by the animals, the amount of MT also increases and it is maximum at 18 h after the last Zn injection was given. Our results corroborate with the observations of other researches who also observed a dose-dependent elevation of cellular levels of MT [46–48]. Also, the concentration of MT in liver, induced by metal supplementation through injection, has been observed to be maximum at 18 h post treatment in various other studies even when 10 mg/kg body weight is given [27, 46]. The half-life of the MT-I is 12.2 ± 0.8 h and MT-II is 21.9 ± 3.0 h, as calculated by pulse-labeling technique [49]. Taken together all the above facts imply that the presence of excess of Zn as in case of present study is not

affecting the time dependent behavior of MT expression or its degradation pattern, although, MT expression was found to be proportional to the supplemented Zn concentration, but not linearly.

The balance between the physiological and pathological functions of zinc is determined by its free intra cellular concentrations [50]. Whenever some reactive compounds (such as heavy metals, toxins, drugs, etc) alter the zinc buffering capacity of cells, it leads to cellular injury because of the interactions of zinc ions with undesirable proteins and the change in redox status [50]. Under these circumstances, the cellular protection is achieved by induction of proteins like MT that can not only sequester excess Zn, but also export it. The interplay between the functions of MT (Zn

sequestration) and Zn transporter (efflux of excess of free Zn ions) is responsible for the regulation of intracellular free zinc levels. The directly proportional relationship between MT expression and the amount of Zn administered in rats suggests that MT is responsible for the initial buffering of Zn ions through their sequestration. The MTF-1 responsible for MT gene expression can sense Zn in nanomolar range through its zinc fingers, whereas classic zinc fingers can do this in the picomolar levels [51]. As soon as, the concentration of intracellular free zinc ion increases, it is sensed by MTF-1, leading to MT protein expression as in the case of present study. The newly synthesized MT will sequestered the free Zn ions and thus, protecting the cells. The effect of zinc exposure on MT content and their role in providing protection against the toxicity of heavy metals like mercury has also been studied [52, 53].

5 Conclusion

With the increase in the dose of Zn from 10 mg/kg body weight to 100 mg/kg body weight, the expression of MT increases though not linearly. As a function of time after last Zn injection, the amount of MT is maximum at 18 h and then it starts decreasing, which is near to its half life as reported earlier in the literature. In presence of excess of Zn beyond hepatoprotective levels, the cellular accumulation of MT as evident from MT expression seems to be responsible for sequestering Zn and maintaining its homeostasis.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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