

Biosynthesis of metallothioneins under cadmium stress, zinc and iron supplementation in liver and kidney of male albino rats

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Abstract— Cells react to stressful environments with a broad range of diverse homeostatic responses. Among an array of responses stress response proteins, including metallothionein (MT) play an important role against metal induced stress. The present study is carried out to know the role of MT in detoxification of cadmium (Cd) toxicity in rats before and supplementation with trace elements such as zinc (Zn) and iron (Fe). Male albino rats were treated orally with Cd at a dose of 1/10th of LD50 / 48h (i.e. 22.5 mg/kg) for 7, 15 and 30 days (d) long sojourn. 15d Cd treated rats were then subjected to trace element supplementations of Zn (12mg/kg) and Fe (40mg/kg) individually and in combination for another 7, 15 and 30d time intervals. After specific time intervals, rats were decapitated and tissues like liver and kidney were isolated and used for metallothionein studies. Purification and quantification of MT protein was carried out by the standard methods. Purified MT protein containing samples were subjected to SDS-PAGE. Clear visible bands were observed in the test tissues approximately at 6.5 KDa against standard low range molecular weight protein marker (Cat. No. M 3546). Further, MT protein levels were significantly elevated in the test tissues during Cd treatment and also after supplementation with Zn and / or Fe. Maximum MT protein synthesis was observed in 30d rat kidney under combined supplementation of both Zn and Fe. Thus, tissues that contain an excess amount of MT are resistant to Cd toxicity.

Index Terms— Cadmium, zinc and iron supplementation, metallothionein, Purification and quantification, liver, kidney, rat.

1 INTRODUCTION

MT is a molecular weight (6-7 kDa), Cysteine (Cys) - rich and stress response protein with a high affinity for divalent heavy metals such as Cd^{1,2}. MT protein that is induced by other divalent metal cations (e.g. mercury, cadmium,) protects essential cellular functions³ and enhances the survival of both cells and whole organisms that are exposed to toxic heavy metals. These MTs are ubiquitously present in a large variety of prokaryotic and eukaryotic species as well as in all mammalian organs and tissues examined so far both in animal and plant kingdom and are increasingly being demonstrated to play a vital role in metal homeostasis⁴.

In mammals, four isoforms of MT have been reported (MT-I, II, III and IV). MT-I and II are found in the liver and kidney tissues. MT - III has been detected in mouse and human brain, and MT - IV has been found in certain stratified epithelia^{5,6,7,8}. The biological function of MT is likely related to the physiologically relevant metals (Zn, Cu and Fe) that this protein binds. In mammals, among MT isoforms, MT-I is found to bind Zn and Fe under normal physiological conditions. Both Zn and Fe are essential trace elements in biology as components of wide variety of metalloproteins and wealth of enzyme families^{9,10}. Zn is known to be the most effective MT inducer and Fe is categorized as indirect MT inducer¹¹. Fe binds significantly less tightly to MT than Zn¹². The relative affinities of these metal ions to sulfur ligands are well established from inorganic thiolate complexes¹³ MT is primarily synthesized on free polysomes¹⁴. Based on their synthesis, MT protein has been often considered to function exclusively as an intracellular protein. While MT lacks the signal peptide sequences or other protein trafficking signals that would result in proteins entering the traditional secretory pathway, it is nevertheless detected in serum, urine, pancreatic secretions and other bio-

logical fluids as well as in bronchoalveolar spaces, liver sinusoids and other extra cellular spaces. This pool of extracellular MT may originate from necrotic cell death that may accompany some forms of stress but it is also possible that MT may be selectively released from stressed cells by non-traditional secretory pathway¹⁵. Regardless of its origin, there is compelling evidence that the pool of extracellular MT protein has interesting roles of its own to play. One possible role of extracellular MT protein is as a distribution mechanism for both essential and toxic heavy metals. Another aspect of MTs extracellular influences is its influence on immune capacity. Recent studies have produced strong evidence to support the idea that the MT functions as a metal chaperone for the regulation of gene expression and for synthesis and functional activity of proteins such as metalloproteins and metal-dependent transcription factors^{16,17}. MT could thus serve as a reservoir of essential metals such as Zn, Se, Fe and Cu. This might dictate dual functions of MTs:

1. Preventing metal toxicity under over load conditions and
2. Donating metals to apo-metalloproteins under physiological conditions.

A large amount of subsequent work has been shown that MT to serve in many cell types in the management of essential divalent metal cations, to interfere with the toxic effects of xenobiotics, heavy metals and free radicals. It also serves as a regulator of specific transcription factors¹⁴. As a consequence of these various activities, MT synthesis has an impact on developmental processes and on the cellular responses to stressful conditions under heavy metal burden¹⁸. In vitro studies indicate that the transfer of metals from MT to an acceptor is possible and GSH has been known to facilitate such a transfer. Most of these metal transfer studies have focused on MT regu-

lation of Zn and Fe homeostasis.

MTs are normally present in animal tissues in only trace amounts. However, the administration of physiologically relevant metals (Zn and Fe) can increase the concentration of MT, which involved in homeostasis of essential metals and provide detoxification mechanism against Cd toxicity¹⁹. Romero et al.,²⁰ demonstrated that the presence of MT in the body of organisms as an indicator of heavy metal contamination of natural ecosystems. Because of the structure of MT is highly conserved, it is likely to perform an evolutionarily conserved function, rather than a function that solves the cells problem with relatively recent pollution.

Though, few studies were oriented towards MT and Cd^{21,22,23} studies related to the interactions between Zn, Fe and Cd with respect to MT is poorly understood. Indeed, there is no sufficient work on MT with regarding to supplementation of trace elements Zn and Fe to reduce the Cd induced toxicity in rats. Hence, the present work was carried out to determine whether the MT synthesis is helpful in reducing the Cd induced oxidative stress in male albino rats under Cd stress and also with supplementation of essential trace elements Zn and / or Fe.

2. MATERIALS AND METHODS

2.1 Chemicals

Cd as cadmium chloride (CdCl₂), Zn as zinc chloride (ZnCl₂), and Fe as ferric chloride anhydrous (FeCl₃) were purchased from Merck (Dormstadt, Germany). All other chemicals which were used in the present study were obtained from the standard chemical companies like Sigma Chemical Co. (St Louis, MO, USA) and SD Fine Chemicals, India. The chemicals used in this study were of the highest purity.

2.2 Animals

Three-months-old Wistar strain male albino rats weighing 180 ± 20 g were chosen for the present study. The animals were obtained from Sri Venkateswara Traders, Bangalore, Karnataka, India and were kept in stainless steel mesh cages, housed under standard laboratory conditions (23 ± 2°C, 50 ± 20% relative humidity, 12-h light-dark cycle) with standard rat chow (Sai Durga Feeds and Foods, Bangalore, India) and drinking water ad libitum. The rats were acclimatized to the laboratory conditions for 10 days. The protocol and animal use has been approved by the Institutional Animal Ethics Committee (Resol. No. 10(ii)/a/CPCSCA/IAEC/SVU/AUR-JO dt 22-12-2008), Sri Venkateswara University, Tirupati, Andhra Pradesh, India.

2.3 Experimental design

After acclimatization, the rats were divided into two groups, namely control and experimental. Each group contains 6 animals. Control rats received only deionized water without Cd. The experimental rats were treated with Cd as CdCl₂ at a dose of 1/10th LD₅₀/48h, that is, 22.5 mg/kg body weight over a period of 7, 15, and 30 days (d) time intervals. Then 15d Cd-treated rats were divided into three groups. Group I received supplementation of Zn (12mg/kg) for 7, 15, and 30d. Group II received Fe supplementation (40 mg/kg) and Group III animals were supplemented with both Zn and Fe at the above said doses for 7, 15, and 30d long sojourn.

2.4 Isolation of tissues

After specific time intervals, the control and experimental rats were decapitated and tissues such as liver and kidney were quickly isolated under ice cold conditions and weighed to their nearest mg using Shimadzu electronic balance. After weighing, tissues were immediately used for the metallothionein studies and were stored at -80°C for future use.

2.5 Isolation and Purification of MT

The stress protein MT under Cd administered male rats and trace elements (Zn and Fe) supplemented rats was isolated at the specified time intervals and purified by adopting following methods. The initial isolation method of MT was based on those of Fowler et al.,²⁴. Fresh tissues such as live and kidney were taken and homogenates prepared in equal volumes of 10mM Tris-HCl buffer pH 7.4 containing 1mM phenyl methyl sulfonyl-fluoride (PMSF) with 0.1mM β-mercapto ethanol to prevent SH group oxidations. The homogenate was centrifuged at 27,000g for 15 minutes and the resulting supernatant was again centrifuged at 105,000g for 60 minutes at 4°C without heat treatment step. The clear supernatant was subjected to initial purification process.

Gel Filtration

Above supernatant fractions of each tissue was applied to a column of Sephadex G-75 (5 x 50cm) equilibrated with 10 mM Tris-HCl buffer (pH 7.4). The column was eluted with 10 mM Tris-HCl at a rate of 50 ml/hr. Major Cd containing fractions were pooled and monitored in AAS and again purified by Ion exchange chromatography.

Ion exchange chromatography

Ion exchange chromatography purification was carried out with DEAE-32 CELLULOSE by using Overnell and Coombs²⁵ method. A glass column of (3 x 40cm) was used for packing the anion exchanger and rubber tubing's were washed thoroughly and checked for air block. Major Cd - binding fractions were pooled and applied to a column of DEAE - Cellulose pre equilibrated in 10 μM Tris-HCl-buffer (pH 7.4). The column was eluted with a linear gradient of 10-350mM Tris-HCl buffer (pH 7.4). Fractions of 20 ml were collected and monitored for Cd in AAS (Shimadzu - AA 6300) and A 250. The main single peak fraction 'Coincide' with high A250 was pooled and further purification was carried out.

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2.6 Quantification of MT

The MT protein content of the tissues was quantified by the method of Lowry et al.,²⁶.

3. DATA ANALYSIS

The data was subjected to statistical analysis such as mean, standard deviation (SD) and analysis of variance (ANOVA) using standard statistical software, Statistical Package for Social Sciences (SPSS; version 16). All values are expressed as mean \pm SD of six individual samples. Significant differences were indicated at $P < 0.05$ level.

4. RESULTS

Tissues such as liver and kidney were isolated from control and experimental rats. The test tissues were subjected to purification and quantification of MT protein as described by Overnell and Coombs²⁵. The supernatants thus obtained after centrifugation were subjected to gel filtration by using Sephadex G-75 column and DEAE - Ion Exchange chromatography. The fractions were collected from the samples after performing Ion Exchange chromatography and were characterized by using SDS-PAGE. The characterization of MT was based on purification and quantification of MT protein from the tissue samples which were already standardized in our laboratory²⁷.

After staining and de-staining, the Gel electrograms were examined under Alpha Image Analyzer (Alpha Innotech) in liver and kidney of control and experimental rats. The bands were visible and approximately with a molecular weight of 6.5 KDa in liver (Plate: 1-4) and kidney (Plate: 5-8) of control and experimental rats against a standard low range molecular weight protein marker (Cat. No. M 3546) obtained from Sigma Chemical Co. (St Louis, Mo, USA). The visibility of clear bands in all gel electrograms of test tissues at 6- 7 KDa indicates the presence of MT, as the MT protein molecular weight was estimated approximately to be 6-7 KDa by Overnell and Coombs²⁵ and Kagi et al.,²⁸. The presence of bands in control and experimental animals around 6-7 KDa clearly indicates the expression of MT protein in the test tissues.

After purification and characterization, the quantification of MT protein content was carried out in both liver kidney tissues of rat in control and experimental rats. The synthesis of the metallo protein, MT in Cd treated rat liver and kidney both before and after supplementation with Zn and / or Fe were tabulated (Table: 1 - 4). The significant increase in the synthesis of MT protein levels in Zn and / or Fe supplemented rats over Cd treated rats as well as control are statistically significant at the level of $p < 0.05$.

Results revealed that MT levels were profoundly increased in both liver and kidney of Cd treated rats at all the time intervals when compared to the controls. Elevation in the MT protein synthesis may indicate its role in the detoxification of heavy metals and also scavenging of ROS. 30d Cd treated rat kidney showed maximum synthesis of MT protein ($15.095 \pm 0.454 \mu\text{g/g}$ wet weight of the tissue) followed by 30d Cd treated rat liver ($12.013 \pm 0.282 \mu\text{g/g}$ wet weight of the tissue).

Table - 1

MT Protein ($\mu\text{g} / \text{g}$ wet weight of the tissue) levels in the selected tissues of Cd treated Rats.

Tissue	Control	7d Cd	15d Cd	30d Cd
Liver	$7.245^a \pm 0.325$	$9.152^b \pm 0.220$	$10.028^c \pm 0.571$	$12.013^d \pm 0.282$
Kidney	$8.025^a \pm 0.155$	$10.145^b \pm 0.471$	$11.189^c \pm 0.406$	$15.095^d \pm 0.454$

All values are expressed as Mean \pm SD of 6 individual samples. Means of the same row followed by different letter (s) differ significantly ($p < 0.05$)

Table - 2

MT Protein ($\mu\text{g} / \text{g}$ wet weight of the tissue) levels in the tissues of Cd treated rats after supplementation with Zn.

Tissue	15d Cd	7d Zn	15d Zn	30d Zn
Liver	$10.028^a \pm 0.571$	$11.839^b \pm 0.314$	$13.010^c \pm 0.314$	$14.827^d \pm 0.313$
Kidney	$11.189^a \pm 0.406$	$13.832^b \pm 0.327$	$15.138^c \pm 0.342$	$17.141^d \pm 0.363$

All values are expressed as Mean \pm SD of 6 individual samples. Means of the same row followed by different letter (s) differ significantly ($p < 0.05$)

Table - 3

MT Protein ($\mu\text{g} / \text{g}$ wet weight of the tissue) levels in the tissues of Cd treated rats after supplementation with Fe

Tissue	15d Cd	7d Fe	15d Fe	30d Fe
Liver	$10.028^a \pm 0.571$	$11.658^b \pm 0.337$	$12.138^c \pm 0.381$	$13.886^d \pm 0.324$
Kidney	$11.189^a \pm 0.406$	$13.211^b \pm 0.309$	$13.982^c \pm 0.292$	$16.579^d \pm 0.342$

All values are expressed as Mean \pm SD of 6 individual samples. Means of the same row followed by different letter (s) differ significantly ($p < 0.05$)

Table - 4

MT Protein ($\mu\text{g} / \text{g}$ wet weight of the tissue) levels in the tissues of Cd treated rats after supplementation with both Zn and Fe

Tissue	15d Cd	7d Zn and Fe	15d Zn and Fe	30d Zn and Fe
Liver	$10.028^a \pm 0.571$	$12.265^b \pm 0.339$	$13.617^c \pm 0.426$	$15.081^d \pm 0.386$
Kidney	$11.189^a \pm 0.406$	$14.110^b \pm 0.303$	$15.717^c \pm 0.468$	$17.481^d \pm 0.313$

All values are expressed as Mean \pm SD of 6 individual samples. Means of the same row followed by different letter (s) differ significantly ($p < 0.05$)

After supplementation with Zn and Fe either individually or in combination to 15d Cd treated rats, the MT levels were highly elevated in both liver and kidney during all the time intervals. Maximum MT protein synthesis was found in 30d rat kidney under combined supplementation of Zn and Fe ($17.481 \pm 0.313 \mu\text{g} / \text{g}$ wet weight of the tissue) than the liver under 30d supplementation of both Zn and Fe mixture ($15.081 \pm 0.386 \mu\text{g} / \text{g}$ wet weight of the tissue). Moderate increment in MT protein synthesis was found in 30d Zn supplemented rat kidney and liver ($17.141 \pm 0.363 \mu\text{g} / \text{g}$ wet weight of the tissue and $14.827 \pm 0.313 \mu\text{g} / \text{g}$ wet weight of the tissue re-

spectively). While in 30d Fe alone supplementation both test tissues showed low level of increment in MT protein content ($16.579 \pm 0.342 \mu\text{g} / \text{g}$ wet weight of the tissue in kidney and $13.886 \pm 0.324 \mu\text{g} / \text{g}$ wet weight of the tissue in liver) than the other modes of supplementation.

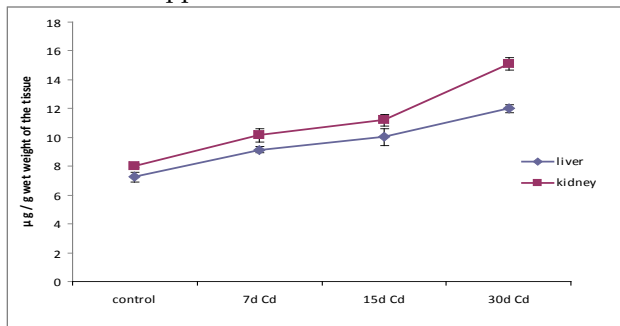


Fig. 1: MT Protein ($\mu\text{g} / \text{g}$ wet weight of the tissue) levels in the selected tissues of Cd treated Rats.

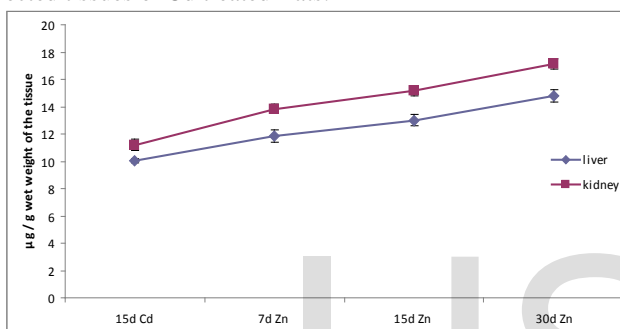


Fig. 2: MT Protein ($\mu\text{g} / \text{g}$ wet weight of the tissue) levels in the tissues of Cd treated Rats after supplemented with Zn.

From the present investigation, it is clear that the MT protein synthesis was significantly high during combined supplementation of Zn and Fe than the individual supplementation of Zn and Fe. The elevation in MT synthesis indicates its role in detoxification of heavy metal Cd and also in scavenging of ROS, which were generated by Cd burden in the liver and kidney tissues.

5. DISCUSSION

In recent years, Cd has been recognized as one of the most toxic environmental and industrial pollutant due to its ability to induce severe alterations in various organs and tissues. One of the tissue protection mechanisms against these toxic effects of Cd is MT synthesis²⁹. MTs are cytoplasmic proteins that sequester certain divalent metal cations and are considered as primary cellular defense against the toxic transition metal Cd. In the present investigation, MT protein purification and quantification were carried out in both liver and kidney tissues of male albino rat and it was suggested that both kidney and liver are main target organs for Cd³⁰. The changes in the MT levels in liver and kidney of Cd exposed rats might reflect the absorption kinetics of the Cd in selected tissues^{7,31}. MT protein levels were significantly increased in Cd treated rats over controls in the present investigation which indicates that Cd exposure induces MT synthesis in liver and kidney of rats. The results are in consonance with earlier reports^{23,29}. Previous

studies of Lu et al.,^{32,33}, Chaumont et al.,³⁴, Chen et al.,⁷ and Kukner et al.,²⁹ revealed that the MT synthesis was high in Cd exposed workers. In another study, Kang³⁵ also reported that the cells contain an excess amount of MT that are resistant to Cd toxicity.

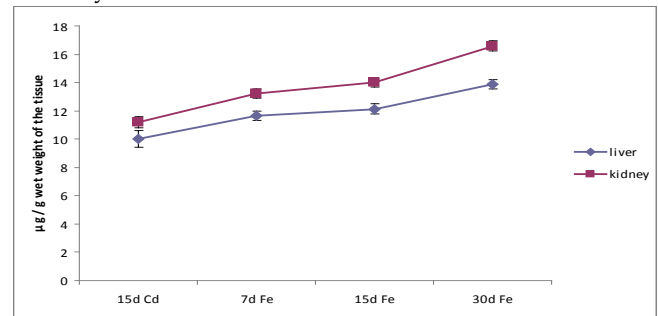


Fig. 3: MT Protein ($\mu\text{g} / \text{g}$ wet weight of the tissue) levels in the tissues of Cd treated Rats after supplementation with Fe.

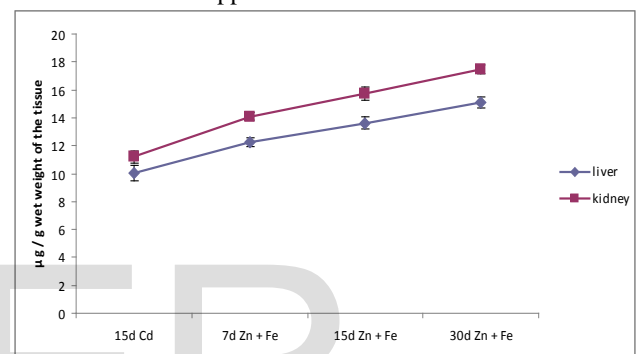


Fig. 4: MT Protein ($\mu\text{g} / \text{g}$ wet weight of the tissue) levels in the tissues of Cd treated Rats after supplementation with Zn and Fe.

Results revealed that the high levels of MT protein synthesis were found in liver and kidney of rats during supplementation with essential trace elements like Zn and Fe over Cd treated as well as control rats (Fig. 1 – 4). Electrophoretic analysis of MT in the present investigation also shows the significant induction of MT protein in the liver (Plates: 1 to 4) and kidney (Plates: 5 to 8) both before and after supplementation with Zn and Fe to Cd treated rats. To protect from heavy metal toxicity, organisms synthesizes more MT protein as it involves in the homeostasis of essential metal ions (Zn and Fe), detoxification of heavy metals (Hg and Cd), protection against oxidative damage through scavenging of ROS, cell proliferation and apoptosis^{36,37,38,39,40,41}. However, the occurrence of MT protein in the control rats in the present study indicates the ubiquitous nature in the organism of non-polluted environments^{42,43,44,45,46}. Increased synthesis of MT has been thought to produce antioxidant effect against ROS in Cd and other heavy metal intoxications⁴⁷.

More synthesis of MT protein was found in kidney tissue than liver in all the modes of supplementation with Zn and / or Fe as well as Cd treatment. A notable induction of MT protein under Cd over load in the kidney of the present study suggests that kidney as a "Critical Organ" to Cd toxicity and also is in agreement with the earlier reports of Ryan et al.,⁴⁸ and Hashem et al.,⁴⁹.

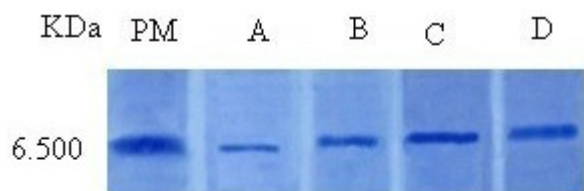


Plate - 1: SDS-PAGE of Liver
PM: Protein Marker
A: Control liver
B: 7d Cd treated liver
C: 15d Cd treated liver
D: 30d Cd treated liver

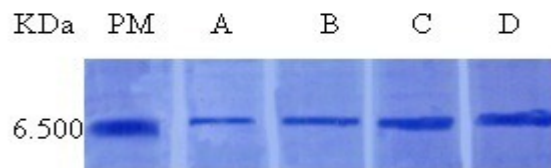


Plate - 5: SDS-PAGE of Kidney
PM: Protein Marker
A: Control kidney
B: 7d Cd treated kidney
C: 15d Cd treated kidney
D: 30d Cd treated kidney

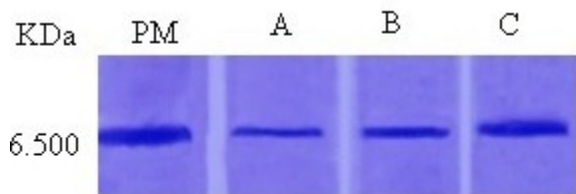


Plate - 2: SDS-PAGE of Liver
PM: Protein Marker
A: 7d Zn supplemented liver
B: 15d Zn supplemented liver
C: 30d Zn supplemented liver

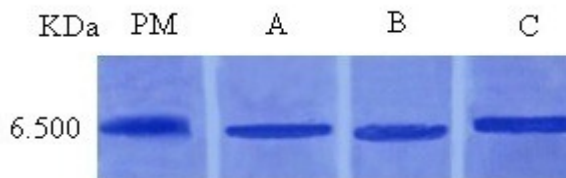


Plate - 6: SDS-PAGE of Kidney
PM: Protein Marker
A: 7d Zn supplemented kidney
B: 15d Zn supplemented kidney
C: 30d Zn supplemented kidney

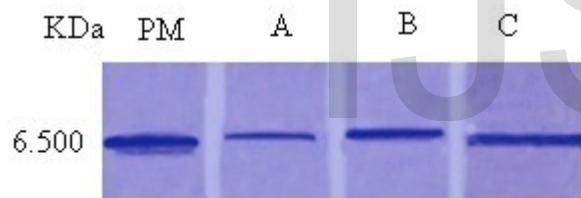


Plate - 3: SDS-PAGE of Liver
PM: Protein Marker
A: 7d Fe supplemented liver
B: 15d Fe supplemented liver
C: 30d Fe supplemented liver



Plate - 7: SDS-PAGE of Kidney
PM: Protein Marker
A: 7d Fe supplemented kidney
B: 15d Fe supplemented kidney
C: 30d Fe supplemented kidney

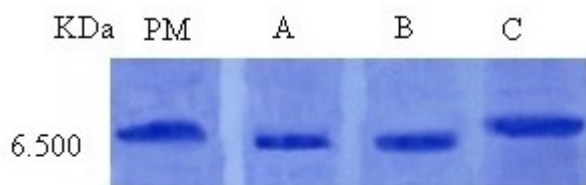


Plate - 4: SDS-PAGE of Liver
PM: Protein Marker
A: 7d Zn + Fe supplemented liver
B: 15d Zn + Fe supplemented liver
C: 30d Zn + Fe supplemented liver

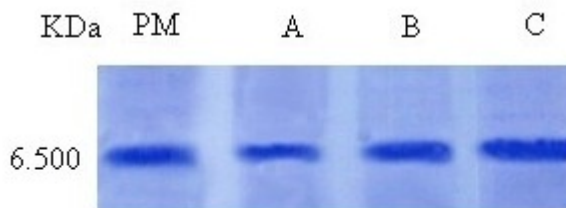


Plate - 8: SDS-PAGE of Kidney
PM: Protein Marker
A: 7d Zn + Fe supplemented kidney
B: 15d Zn + Fe supplemented kidney
C: 30d Zn + Fe supplemented kidney

Liver and Kidney are the two important organs which play critical role in a range of physiological functions including MT synthesis. The present data shows low level of hepatic MT concentration than renal MT concentrations. Many reports suggested that ingestion of Cd is absorbed and transported to plasma where it binds with albumin to form Cd-albumin complex⁵⁰ via pulmonary or gastro intestinal route. Cd-albumin is absorbed predominantly by liver and Cd is released from the albumin in the liver tissue. The released Cd induces synthesis of MT in the liver and most of the Cd is bound to MT^{51,52}. As liver is the first site of Cd bioaccumulation, where Cd binds with MT⁵³, the Cd-MT complex transported to kidney tissue might have caused MT increase in the renal tissue. Several authors also reported that MT protein is induced in liver under Cd intoxication, as it is the first site of accumulation of Cd and plays a vital role in the formation of Cd-MT complex and from there the complex is transported to kidney^{7,31,50,54}. A small proportion of Cd-MT complex will be released into plasma as it is having low molecular weight and it will be efficiently transported through the glomerular membrane and reaches the tubular fluid in kidney, where it will be taken up by pinocytotic vesicles in the brush border of the proximal tubules and transported into lysosomes and actually here the Cd is released from Cd - MT complex. The released Cd may cause adverse effects in the renal tissue and further it may get eliminated from the body^{55,56}. Park et al.,¹¹ and Liu et al.,⁵⁷ reported the protective role of MT against Cd toxicity by using wild type and MT null mice to determine LD₅₀ for Cd and it was found that the LD₅₀ value for the wild type to be 6.9 folds higher than MT null mice suggesting MTs induction against Cd intoxication. Interestingly, one of the important findings of the present study is that supplementation of Zn and Fe either alone or in combination significantly induced MT in liver and kidney of rats over the Cd treated rats. The magnitude of MT concentration in liver and kidney of rats were as follows: Zn + Fe > Zn > Fe > 15dCd.

Zn is a physiologically important metal and the most abundant metal bound to constitutive MT. Zn provides essential structural and catalytic functions to a wide variety of proteins. More than 300 different enzymes depend on Zn for proper protein folding and biological function. It is also crucial in the regulation of gene expression because numerous transcription factors have "zinc finger motifs" that are maintained by Zn. It is believed that MT plays an important role in Zn metabolism and is popularly known as reservoir of Zn. Previously it has been reported that transgenic mice suggested a role for MT-1 and MT-2 isoforms in Zn metabolism. Results have proposed that MT on the one hand protects the cell from high Zn levels by the production of Zn - MT and that on the other hand, it offers a driving force for Zn-uptake by the transient production of apo-metalloprotein (apo-MT)⁵⁸. MTs could serve as a reservoir of Zn while preventing metal toxicity and might

be involved in Zn transfer to newly synthesized apo-MTs including enzymes and Zn finger proteins (transcription factors, signaling, and adapter molecules). Zn-MT rescues the function of Cd - substituted tramtrack, a zinc finger transcription factor^{59,60,61,62}. When Cd displaces Zn in tramtrack, this protein loses DNA binding activity. Incubating Zn - MT with Cd tramtrack in vitro allows the exchange of Cd and Zn, with the transcription factor regaining its DNA binding activity. Hence, Zn-MT may rescue zinc finger proteins from inactivation by other metals.

It has been demonstrated that the cluster structure of Zn - MT provides a chemical basis by which the Cys ligands having oxido-reductive properties³⁵. The structure allows for thermodynamic stability of Zn in MT while permitting Zn to retain kinetic liability. Importantly, Zn release from MT is modulated by GSH and GSSG. GSH inhibits Zn release in the absence of GSSG, indicating MT is stabilized at relatively high cellular GSH concentrations. The presence of GSSG or any other oxidizing agent, results in a release of Zn that is synergistically increased by GSH. The rate of Zn release depends linearly on the amount of GSSG (i.e., the more oxidative redox state becomes, the more efficiently Zn is released from MT)⁶³. Thus, it is apparent that Zn maintains the homeostasis of MT thereby protects the cells from heavy metal induced toxicity.

In the present study, Cd treatment significantly induces MT in selected tissues. Earlier it has been hypothesized that immediately after uptake of Cd into the cell, the reactions of the cell begin to shift to the right resulting in an increase in transcription. Cd displaces Zn that is bound to cellular protein increasing the amount of free intracellular Zn. As the intracellular concentration of Zn increases, it binds to the inhibitor which dissociates from Metallothionein transcription factor (MTF)-1 allowing MTF-1 to bind to the Metallothionein responsive element, thus enhancing the initiation rate of transcription. Another driving reaction is that occurring at the same time is the complexing of Cd to cellular proteins which decreases the cellular protein binding sites available for Zn binding contributing to the increase in the concentration of free Zn within the cell. As MT protein is produced, both Cd and Zn are bound to the newly synthesized MT and the pool of free Cd and Zn decreases. This decrease of free Zn within the cell increases the available pool of inhibitor that then binds to MTF-1. Once the MTF-1 inhibitor complex is formed, the rate of transcription returns to basal levels.

Fe is another essential trace element that plays an important role in MT and hemoglobin synthesis and also in redox reactions. It is an essential nutrient to almost all organisms^{64,65} and plays an essential role in biological processes. It is also a vital component of several enzymes and proteins⁶⁶. Fe is a key molecule in the CAT and induces MT either indirectly or by way of antioxidant response elements^{67,68}. In the present study, the supplemented Fe influenced the expression of MT in both the test tissues of rat under experimentation. The mechanism of MT protein induction by Fe is not elucidated.

Quantification of MT protein revealed that the kidney tissue showed more expression of MT than the liver tissue under Fe alone supplementation at all the time intervals of experimentation. Yasutake and Hirayama⁶⁹ reported that the supplementary Fe enhances MT turnover in the kidney than liver, alt-

though the mechanisms of such processes are not understood at present. However our studies suggest that the increase of MT protein level in both the test tissues is probably mediated by the differential expression of MT gene. Interestingly, supplemented Fe was found to elevate the MT protein expression in the liver and kidney tissues of Cd treated rats, providing further evidence of the ameliorative effects of Fe supplementation against Cd induced stress response in Wistar strain male albino rats. Similar findings were reported by Kwong et al.,⁷⁰ in rainbow trout under Cd stress with intake of dietary Fe. It is well known that Zn and / or Fe provides protection against Cd induced alterations through the induction of MT either directly or indirectly^{71,72,73} activation of antioxidant defense system and decreases the ROS generation^{74,75,76,77}.

Functionally, MT is involved not only in heavy metal detoxification and homeostasis of essential trace elements (Zn and Fe), but also in scavenging the ROS during oxidative damage^{16,78}. Induction and synthesis of MT in the tissues by oxidative and chemical stress and involves in the protection of tissues against Cd - induced toxicity in various animals as well as human beings have been well documented^{7,29,32, 33,34,42}. Similarly Hosokawa et al.,⁷⁹ and Cai et al.,⁸⁰ also reported the protective role of MT protein in rats against Cd mediated nephrotoxicity, allogeneic stimuli - mediated oxidative testicular damage and N-nitrosodimethylamine - mediated oxidative and hepatotoxic effects.

The high metal - inducibility of the MT have been linked with the role in heavy metal detoxification and sequester them into biologically inactive forms. MT protein may protect against Cd toxicity by: 1. reducing the Cd uptake into cells. 2. sequestration of Cd within the cells and 3. enhanced Cd export out of the cells. The mechanism of MT in cellular defense against Cd toxicity includes sequestration of free Cd to form the inactive Cd-MT complex and alteration of the intracellular distribution of Cd.

ROS (including superoxide, hydroxyl radicals and hypohalous acids) formed by infiltrating cells or by resident glomerular cells have been implicated in renal dysfunction⁸¹. Accumulation of ROS in the tissues is controlled by enzymatic antioxidants such as SOD, CAT and GST etc., and also by non-enzymatic intracellular protein MT, a family of stress - induced protein with diverse physiological functions, including protection against Cd toxicity. In vivo studies have also shown that both MT-1 and MT-2 are capable of scavenging free radicals, but the MT-1 appeared to be a superior scavenger of superoxide⁸¹. This suggests that the induction of MT protein synthesis in Zn and / or Fe supplemented rats over Cd treated rats indicates the involvement of MT protein in the scavenging of ROS and thus it provides support to the cells against oxidative stress under Cd burden^{39,82,83,84,85}.

Cellular accumulation of MT depends on the gene expression and protein degradation. Both depend largely on the availability of cellular concentration of Zn and Fe derived from the supplementary Zn and Fe¹⁶. Zn and Fe may protect -SH groups from oxidation or may limit the redox active metal content of tissues. Many have advanced the hypothesis that supplemental Zn and Fe provides antioxidant protection through its powerful induction of MT gene regulation^{16,86,87}. The observations in the present study provide new insights

into the correlation between antioxidant defense enzymes (ADE) and MT induction in the control as well as experimental rats.

4 CONCLUSION

Based on the overall discussion it may concluded that the mixture of Zn and Fe supplementation was more effective in the MT protein synthesis as well as in reducing the Cd body burden in the tissues than the individual supplements of Zn and Fe.

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