Effect of Cadmium Chloride and / or Vitamin "C" on Metallothionein Gene Expression

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Abstract: Heavy metals including cadmium represent environmental hazards and a great problem as these heavy metals lead to a great public risks for human, animal and poultry health**.** Metallothionein (MT) is a low molecular weight metal binding protein; it plays an important role in providing protection from cadmium toxicity. Vitamin C is one of the most important antioxidants that is used for prophylaxis and treatment of cadmium toxicity. The current study included 24 Albino rats and divided into four groups, each one includes six rats . First group was kept as controls (CON). The second one was given only vitamin C (VIC). The third was given only cadmium chloride $Cdcl_2$ (CAD).The last group was given both vitamin C and $Cdcl_2$ (VIC+CAD). Metallothionein 1 and 2 mRNA expressions were measured in liver and kidney tissues by SYBR green relative quantitative Real time PCR in all samples. The $Cdcl_2$ increases the expression of both MT1 and MT2 mRNA in liver by 4.421 and 3.827 fold and in kidney tissues by 1.095 and 1.203 respectively. The study also demonstrates that vitamin C decreases the expression of MT1 and MT2 in kidney tissues by 0.214 and 0.191 fold respectively and has no significant effect on liver tissues. It was suggested that MT and MT2 mRNA act as biomarkers of cadmium toxicity. Vitamin C had a protective role against cadmium through its antioxidant effect and that appeared through decreasing the expression of both MT1 and MT2 in liver and kidney tissues.

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1. Introduction

Heavy metals present in soils represent environmental hazards and a great problem as these heavy metals lead to a great public risks for human, animal and poultry health (Pinot *et al.* 2000; Oprea *et al.* 2010). Cadmium is widely distributed in the earth's crust at an average concentration of about 0.1 mg/kg. However higher levels may accumulate in sedimentary rocks and marine phosphates often contain about 15 mg Cadmium/kg (Zubillaga, 2008).Marine waters contain about 0.15 mg/liter fresh water less than 1 ppb, and air about 0.002-0.02 µg/m^3. Food intake varies but the mean is thought to be about 50-60 µg/day (Cabrera *et al.,* 1998). Kidneys and liver are considered the most susceptible organs in the case of exposure to cadmium (Ryan *et al.,* 2000). Metallothionein (MT) is a low molecular weight (6000 to 7000 daltons) metal binding protein, the amino acid content of which is approximately 30% cysteine residues. This high content of cysteinyl thiol groups accounts for the high metal affinity of MT (Brammell*,* 2010). MT appears to function, in part, in cellular defenses against metal toxicity. In particular, MT appears to play an important role in providing protection from cadmium toxicity. It is possible that reduced levels of MT gene expression may lead to an increase in a tissue's susceptibility to cadmium carcinogenesis (Lui *et al.* , 2009). Furthermore, cadmium potentially induces oxidative stress. (Satarug and Moore, 2004). The metabolism and excretion of heavy metals depend upon the presence of antioxidants (Patrick, 2003). Antioxidants are substances that retard or prevent deterioration, damage caused by free radicals oxidant (Bolkent *et al.* , 2008)**.**Vitamin C is an aqueous phase antioxidant has been established for many decades and has several important roles. It modulates intracellular status through maintaining sulphydryl compounds, including glutathione, in their reduced state. Also, it serves to maintain membrane alpha tocopherol and enzyme activities including hepatic mixed function oxidase activity. Ascorbic acid, after being converted to dehydroascorbic acid by free radical reaction, is regenerated via the glutathione enzyme complex (Bolkent *et al.,* 2008)**.**

2. Materials and Methods

Twenty-four Albino rats weighted (100-120g) at the beginning of the experiment. The management was kept constant throughout the experimental period. Rats were divided into 4 groups, each group consisted of six rats . The first group was kept as a negative control group (CON). The second group given 180 mg/Kg B.Wt. vitamin C (therapeutic dose in human) as a positive controls (VIC0. The third group given 1mg/Kg B.Wt. cadmium chloride (CAD) .The fourth

group given 1mg/kg.B.wt cadmium chloride in addition to 180mg/kg.B.Wt. vitamin C (VIC+CAD).

2.1. Tissue handling:

After 3 months from the beginning of experiment, animals were scarified and biopsies of liver and kidney were taken immediately placed in Eppendorf tube and stored at -80 ^{0}C for further processing.

2.2.Total RNA extraction:

Total RNA was extracted using RNA Purification Kit; Jena Bioscience (GmbH, Germany), following the manufacturer instructions and the standard protocol. Fifty µl of the eluted RNA was collected immediately, placed in ice and stored at -20°C for further processing.

2.3. Spectrophotometric quantification of RNA:

To ensure significance, A260 readings should be greater than 0.15.An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per mL. The ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity (Alhusseini *et al*., 2010a), 10 µL of each RNA sample was diluted to 500 µl by D.W PH=7.0 (1:50 dilution).Measurement of the absorbance of the diluted sample in a 1mL cuvette (RNase free) at A260 and A280. Concentration of RNA sample was measured: = 44µg m L ^{1}×A260×dilution factor.A260 and A280 were taken by UV spectrophotometer (Optima SP-3000+, Japan). The ratio of the readings at 260 and 280nm (A260/A280) provides an estimate of the purity of RNA. Pure RNA has an A260/A280 ratio of 1.9-2.3

2.4. Relative Quantitation of m RNA of the respective genes by real time PCR using SYBR green:

The target gene sequences was the catalytic subunit of MT 1&2 ,the calibrator sample is healthy control. The reference gene (housekeeping gene) was β-actin. Relative quantitation R.Q provides accurate comparison between the initial levels of template in each sample, without requiring the exact copy number of the template. Also the relative levels of samples can be determined without the use of standard curves. A singleplex reaction have been used in this study, we did two-step RT-PCR using Maxine RT premix kit (IntRON Bio technology) containing oligodt primer and Reverse transcriptase and Real-time- RCR master mix E_3 (2X) (Geneon). C- DNA synthesis reaction using PCR machine performed at 45°C for 60min and RTase inactivation step at 95°C for 5min. In ABI 7900 (Applied Biosystem.) the real time machine the prepared reaction components were done in 96 well PCR plate using real time cycler conditions of 50°C for 1-2 min, (UNG treatment), 95°C, 1-3 min, (Initial denaturation), followed by 40 cycles of 95°C, 30 sec, 55°C, 1 min and 75°C,30 sec for denaturation, annealing, extension steps respectively. Primer sequence of Mt 1 was 5'-ACT GCC TTC TTG TCG CTTA -3 ' (5'-3' sequence forward) and 5'- TGG AGG TGT ACG GCA AGA CT -3' (5'-3' sequence reverse). Primer sequence of Mt 2 was 5'- CCA ACT GCC GCC TCC ATT CG -3 ' (5'-3' sequence forward) and 5'- GAA AAA AGT GTG GAG AAC CG -3' (sequence reverse). Primer sequence of β - actin as internal control (housekeeping gene) was 5' - CCC ATT GAA CAC GGC ATT G -3'(sequence forward) and 5'- GTA CGA CCA GAG GCA TAC A -3' (sequence reverse). The PCR primers were synthesized by (Metabion international AG, Germany).

2.5. Data analysis:

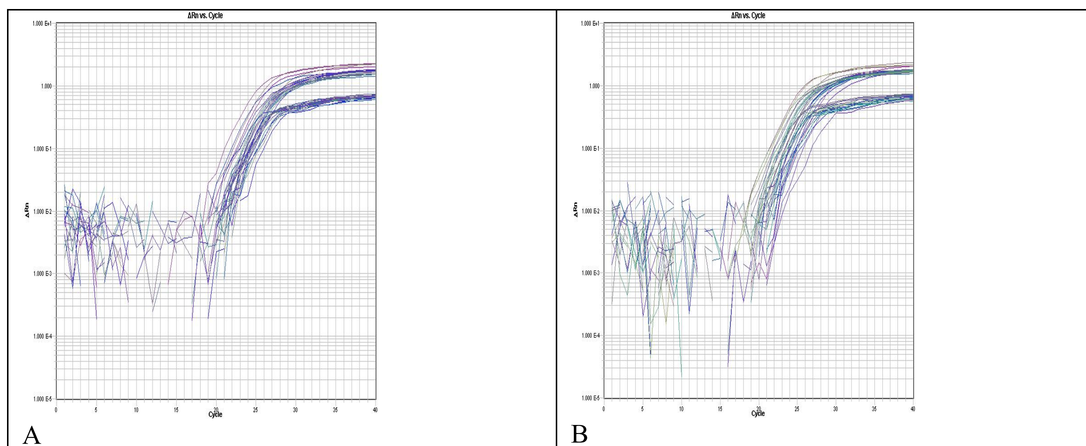
According to the RQ manager program ABI SDS software (ABI 7900), the data are produced as sigmoid shaped amplification plots in which the number of cycle is plotted against fluorescence (when using linear scale). The Threshold Cycle (CT) serves as a tool for calculation of the starting template amount in each sample Figs. 1&2. Because the samples of control group and also samples of treated group are used as calibrators, the expression levels are set to 1. But because the gene expression levels were plotted as log10 values (log10 of 1 is 0), the expression level of the calibrator samples appear as 0 in the graph. (Alhusseini *et al*., 2010b). Because the relative quantities of the MT1&2 gene are normalized against the relative quantities of the endogenous control B-actin gene fold expression changes are calculated using the equation $2^{-\Delta\Delta ct}$ (Livak and Schmittgen, 2001).

2.6.Statistical analyses:

The collected data was tabulated and statistically analyzed. The results are presented as means ± SD (ANOVA test) .All analysis is performed using the statistics package St. for social sciences (SPSS) and Microsoft office Excel is used for data processing and data analysis. Differences are considered as statistically significant for a *p* value less than 0.05

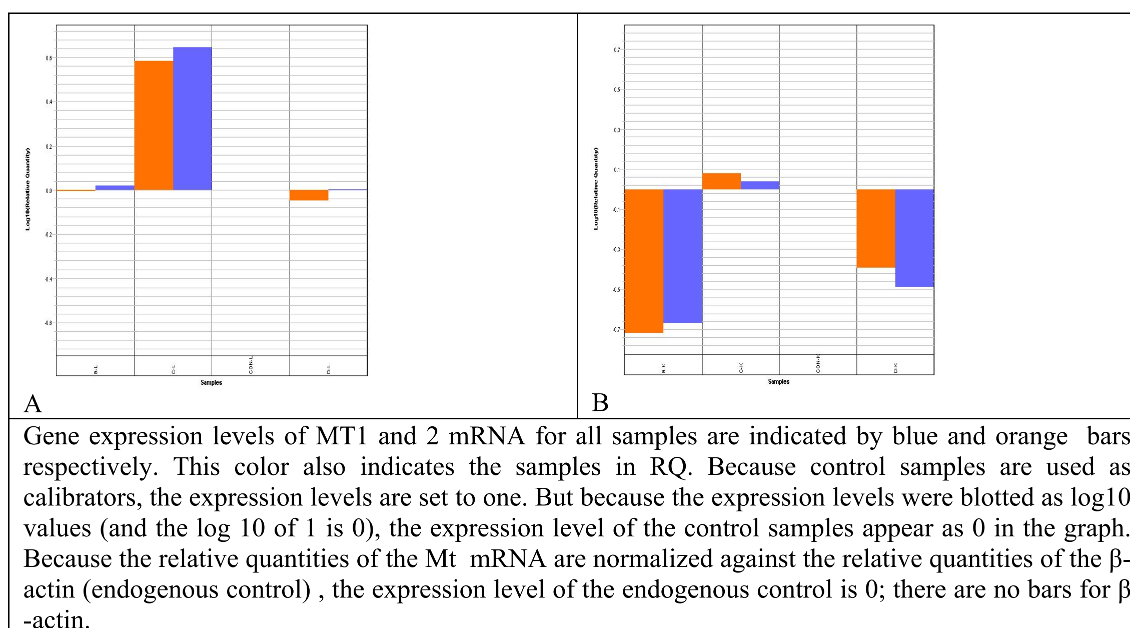
3. RESULTS

The expression of MTs mRNA was significantly increased in liver tissues in CAD group by 4.421 and 3.82 fold increase for MT1 and MT2 respectively as compared to CON group P<0.05. But there in no significant difference among other groups, Table1, Fig1and Fig1,2. As regard to the expression of MTs mRNA in kidney tissues, the study revealed that there was significant decrease of the two types of MTs in both VIC and (VIC+CAD) groups by 0.214 and 0.191 fold decrease respectively as compared to CON group P<0.05 Fig1and Fig2. But in CAD group there are slightly increases in both MT1 and MT2 but not reach to the significant value , Table1, Figs1&.2.



A B

Figure1:Amplification plot curves for detectors metallothionein 1 and 2 genes in liver (A) and kidney (B) tissues among all groups.



A B

Gene expression levels of MT1 and 2 mRNA for all samples are indicated by blue and orange bars respectively. This color also indicates the samples in RQ. Because control samples are used as calibrators, the expression levels are set to one. But because the expression levels were blotted as log10 values (and the log 10 of 1 is 0), the expression level of the control samples appear as 0 in the graph. Because the relative quantities of the Mt mRNA are normalized against the relative quantities of the β-actin (endogenous control) , the expression level of the endogenous control is 0; there are no bars for β -actin.

Figure 2:-Gene expression by Log10 Relative Quantitative levels of Metallothionein 1 and 2 mRNA in liver (A) and Kidney (B) tissues among all groups

Table 1: Gene expression by relative unit of Metallothionin 1 and 2 mRNA in both liver and kidney tissues : The mRNA expressed by Log10 relative units of relative quantitation(RQ)

Tissues	Liver		Kidney	
Target genes	MT1	MT2	MT1	MT2
Groups	Log10 mean± SD			
CON	7.35 ± 0.007^{b}	7.33 ± 0.007^{b}	7.21 ± 0.007^{b}	7.11 ± 0.007^{b}
VIC	7.37 ± 0.007^{b}	7.32 ± 0.008^{b}	6.54 ± 0.007^{d}	6.39 ± 0.008^{d}
CAD	8.00 ±0.009^{a}	7.91 ± 0.008^{a}	7.25 ±0.007^{a}	7.29 ± 0.008^{a}
VIC+CAD	7.31 ± 0.009^{c}	7.28 ± 0.007^{c}	6.72 ± 0.008^{c}	6.75 ± 0.007^{c}
	a: p<0.05		d,c: p<0.05	

4. Discussion

Experience and fundamental similarities in cell structure and biochemistry between animals and humans provide general valid bases for the prediction of likely effects of chemicals on human population (Meyer, 1993).Metallothionein exist not in tissue for various animal species but also in bacteria and plants and are known to detoxify heavy metals (Satoh *et al.,* 2003) . In kidney and liver most of cadmium (76%-87%) In cytosoles was bound to metallothionein as they contain cadmium binding protein which participates in accumulation and distribution of

cadmium so liver and kidney show a tendency to accumulate high level of cadmium (Lind *et al.,* 1997). Our results indicate that $Cdcl_2$ induce Mt expression in liver and kidney of treated rats these are in agreement with Man *and Woo* .(2008) who found that primary hepatocyte culture from silver sea bream *sparussarba* directly exposed to sub lethal level of cadmium *in vitro* showed up regulation of metallothionein mRNA expression. Also, Andreani *et al.* (2011) who showed that metallothionein m RNA concentrations were significantly higher in hepatopancrease of molluscus *scaphorca inaequivalvis* exposed to cadmium. Moreover, Tkatcheva (2007) who demonstrated that 250 ug/ L Cadmium chloride for one hour in rainbotrout *oncorhynohus mykiss* cause induction of metallothionein in kidney.

In the current work ,the expression of MT1 was higher than MT2 in liver while in kidney MT 2 was higher than MT1. These result are in agree with Coogan *et al.* (1994) who found that MT1 gene expression in liver of rat was quite apparent in control tissue and was induced in dose dependant manner 24 hrs following cadmium exposure. On the contrary, Xu-yi *et al*. (2003) observed that MT1 and MT2 the major isoform were found in untreated rat and increased after cadmium treatment and peaked at 3 hours followed by a decline, the induction of MT1 m. RNA was lower than MT2 m. RNA in liver of rat treated with cadmium. Further more, Swiergosz *et al.* (2007) found that MT2 gene expression in liver of bankvoles *clethrionmysglareolus* exposed to heavy metals (Cd, Zn, pb and Fe) was higher than MT1. Gao *et al.* (2009) who revealed that Mt_2 increased in kidney of mandarian fish *siniperca chuasts* after stimulation by cadmium. Similar result was obtained by Wang *et al* .(2009) who reported that expression of MT1 and MT_2 increased significantly in kidney of sprague Dawely rat after exposure to 50 mg/ L cadmim chloride. The findings not agree with Chan *et al.* (2004) who showed that there was high fold induction of MT1 m. RNA in kidney following exposure to cadmium in comman carp *cyprinus carpio*. On the contrary, Sweiergosze *et al*. (2007) who found that MT_2 expression in kidney of Bank vole *clethrionomys glareolus* chronically exposed to heavy metals (cd, Zn, pb and Fe) was lower than MT_1.

The mechanism by which cadmium induce metallothionein in liver and kidney have been proposed by Curtis *et al.* (1998) who showed that cadmium that is imported into cells such as hepatocyte induce synthesis of metallothionein to which over 80% of cadmium binds, cd – MT complexes are released into Blood stream, reabsorbed though the proximal tubules and accumulation in Renal cells. Free cd ions are released by cd- Mt degradation in lysosome in which induce synthesis of metallothionein protein to which they bind again , and accumulation in renal cells in a low toxicity state . More over, Cho *et al.* (2008) who showed that liver was more responsive to heavy metals than kidney. Also they demonstrated that the target organ of cadmium accumulation depends on the periods of administration, most cadmium accumulates in liver after short term exposures, but in kidney in long term exposure. Metallothionein is considered to be primary intracellular component of the protective mechanism and the factor that increase cd toxicity was mainly through inhibition of MT expression Salińska *et al.,* (2012). The transcriptional activation by heavy metals is mediated by several copies of a 15 bp consensus sequence (metal responsive element, MRE) present in the promoter region of all MT genes analyzed to date .Also demonstrated that the mouse MT-I promoter contains six MREs (MRE, a-f) within the first 200 bp 5' of the transcriptional start site. MREa-d confers metal-responsive transcription when tested independently in front of a reporter gene. The ability to mediate metal-activated transcription varies between the different MREs; MREd is the strongest MRE of the MTI promoter. (Larochelle *et al.,* 2001)

Our result revealed that vitamin C had significant effect on MT expression these results were in agreement with Shiarish *et al*. (1993) who demonstrated that ascorbic acid pretreatment on fischer rat increase hepatic metallothionein but not renal metallothionein alone and had no effect on induction of hepatic or renal metallothionein by induction of hepatic or renal metallothionein by cadmium. More over, Berzina and smirnova (2001) who stated that vitamin C prevent the damage and destruction caused by free radical oxidates caused by cadmium due to the effective role of vitamin c in reducing cadmium accumulation and uptake in kidney. Also, they recorded that vitamin C was effective in preventing cadmium accumulation in liver by reducing the uptake of cadmium by the liver and so that it decreased the liver damage. Karabulata *et al.* (2008) showed that vitamin C increase glutathione in kidney of rat given cadmium. Karmakar *et al., (*1998) who reported that hepatic glutathione plays an important role in protection against cadmium toxicity before the onset of metallothionein in rat, and animal in bad condition from interruption of nutrient supply can not be protected against cadmium toxicity even if hepatic metallothionein level is high. Basha *et al.* (2003) showed that glutathione dependant enzymes as well as other antioxidant enzyme function in protection against cadmium toxicity and that these antioxidant provide first line of defense against cadmium before the induction of metallothionein , liver and kidney recorded high activity for all enzyme except glutathione peroxidase (GPX) which was significantly elevated in kidney Also, Tarasub *et al.* (2011)

recorded that kidney contains high activity of gamma glutamyl transpeptidase (GGTP)enzyme which hydrolyze G.S.H this may the explain how vitamin C decrease metallothionein more in kidney .

Conclusion

From the results of this study it could be concluded that Cadmium chloride had effect on metallothionein gene expression on rat producing significant increase metallothionein gene 1 and 2 in

liver and kidney. Metallothionein gene act as a biomarker of cadmium toxicity. Vitamin C had a protective effect against cadmium through its antioxidant action and this effect appear in overcome the effect of cadmium on metallothionein gene 1 and 2 expression and decreased the expression of them in liver and kidney.

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