Role of Glutathione in protection against mercury induced poisoning

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Abstract: Mercury is harmless in an insoluble form, such as mercuric sulfide, but it is poisonous in soluble forms such as mercuric chloride or methylmercury. Mercury is a neurotoxin. Outbreaks of mercuric chloride poisonings have made it clear that adults, children, and developing fetuses are at risk from ingestion exposure to mercury. It is very important and interesting to study the reaction of mercuric chloride and Glutathione as biomarker of Glutathione role in detoxification and conjugation in components (Plasma and Cytosolic Fraction). The effect of mercuric chloride's different concentrations was examined on GSH present in plasma and cytosolic fraction. Decrease in GSH level was dependant on mercuric chloride concentration. The decrease in GSH level of blood components was more prominent with the time of incubation of mercuric chloride. Decrease in the concentration of reduced state Glutathione may be due the interaction of reduced state Glutathione (GSH) and mercuric chloride to form oxidized Glutathione (GSSG) or mercuric-glutathione complex. This change in GSH metabolic status provides information regarding the role of GSH in detoxification of mercuric chloride. The effect of mercury metal on Glutathione in blood components has been discussed in this paper *in vitro* condition as a model for in *Vivo* condition.

Keywords: Mercuric chloride ($HgCl_2$, reduced state glutathione (GSH), plasma, cytosolic fraction (CF), oxidized glutathione (GSSG), Di, thiobis, dinitro-benzoic acid (DTNB).

INTRODUCTION

Mercury poisoning also known as hydrargyria or mercurialism is a disease caused due to the exposure to mercury (Hg) and its compounds. Mercury occurs in many forms, all of them can cause toxic effects in larger doses. Its zero oxidation state Hg^0 exists as vapor or as liquid metal, its mercurous state Hg^+ exists as inorganic salts, and its mercuric state Hg^{2+} may form either inorganic salts or organomercury compounds; the three groups vary in effects. Toxic effects include damage to the brain, kidney, and lungs (Clifton, 2007). Mercury poisoning can result in several diseases, including acrodynia (pink disease), Hunter-Russell syndrome, and Minamata disease (Davidson *et al.,* 2004).

Glutathione (GSH) is main non-protein thiol-compound in mammalian-cells and this tri-peptide has bio-reducing activity (Lomaestro and Malone, 1995). Inside cells, GSH is present in two forms: Reduced state (GSH) and oxidized state (GSSG). Inside cells glutathione is present in reduced state (GSH) and is more than 95% of the total (GSH+GSSG) content. Intra-cellular oxidized Glutathione (GSSG) is present in small quantity but may increase with oxidative-stress or pathological-conditions and reduced glutathione depletion occurs at the same time (Cereser *et al*., 2001). GSH and GSSG act as a thiol redox-couple and have role in gene regulation and intra-cellular signal transduction (Dalton *et al*., 1999). Glutathione in reduced state (GSH) has important roles in cell division, immune-response, signal processes control, programmed cell-death, some xenobiotics and heavy metals detoxification (Ogawa 2005, Meister and Anderson, 1983). GSH has very high redox potential due to which it has anti-oxidant activity and a cofactor for enzymatic reactions that need readily available election pairs (Khan *et al*., 2010). GSH is very reactive from physico-chemical point of view and conjugate to other molecules including the heavy metal ions because of its sulfhydryl moiety (Balendiran *et al*., 2004, Vitecek *et al* 2006). GSH/GSSG ratio gives an early indication of oxidative stress or risk of disease (Cereser *et al*., 2001). Metals toxicity includes geno-toxicity, or carcinogenicity, neuro-toxicity (Flora *et al*., 2006). Glutathione concentration is decreased when reacted with Mercuric chloride, increased the concentration of oxidized-Glutathione (GSSG) (Flora *et al*., 2002). Blood components are having high Glutathione level, have been selected to examine the effect of mercuric chloride on GSH in these components. This study includes the influence of mercury as mercuric chloride on Glutathione level in blood constituents particularly plasma and cytosolic fraction of blood *in vitro* which may accounts towards a model for *in vivo* interaction.

MATERIALS AND METHODS

Materials

L. Glutathione (GSH) (Fluka), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) (sigma), Hydrochloric acid 35% (HCl) (Merck), Sodium hydroxide (Na0H) (Fluka

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AG), mercuric chloride ($HgCl_2$) (Peking Chemical Works, China), Potassium dihydrogen phosphate (Merck), Disodium edetate (Riedel-de Haen AG (Sleeze, Hanover), chloroform (Merck), Ethanol (Merck), sodium chloride (Merck). All the reagents were used as purchased without any further purification.

Different instruments were required, including, pH Meter (Model NOV-210) Nova Scientific Company Ltd., Korea, Oven (Memmert Model U-30), 854 Schwabach (Germany), UV-1601 (Schimadzu), automatic double beam Spectrophotometers (Japan), Graduated Micro-pipette (Scorex Swiss, Finland), Magnetic stirrer, hot plate 400 (England), Sartorius Balance, Eppendolf's tubes (Plastic 101), Siliconazed glass test tubes, Centrifuge (H-200, Kokusan Ensink Company, Japan),

Methods

Preparation of required solutions

100ml of 1mM GSH solution was prepared by dissolving 30.74mg of GSH (molecular weight 307.4) in 0.1N HCl solution. 42.4ml of 0.2M Na0H solution was mixed with 50ml of 0.2M monobasic potassium phosphate solution and diluted upto 200ml with distilled water to prepare 0.2M phosphate buffer pH 7.6 stock solution. 0.9% NaCl solution (Normal saline) was prepared by dissolving 0.90gm (900mg) of the pharmaceutical grade sodium chloride (NaCl) in distilled water and diluting upto 100ml with distilled water. 100 ml of 1mM 5,5-Dithiobis-(2-Nitrobenzoic acid) (DTNB/Ellman's reagent) was prepared by dissolving 39.6mg of DTNB (molecular weight 396.35); in 0.2M buffer (pH 7.6). 1mM isotonic solution of mercuric chloride (molecular weight 271.52) was prepared by dissolving 0.271 gram (271.52 milligram) of $HgCl_2$ in normal saline and was diluted upto 1000ml with 0.9% NaCl solution. To obtain 0.5M disodium edetate (EDTA-2Na, molecular weight 372.2) 1.861gm of it was dissolved in 10ml of distilled water). Then 1 ml of it was diluted upto 100 ml with distilled water to obtain 5mM disodium edetate solution. For to prepare 80 ml of 3:5 Chloroform:Ethanol mixture, 30 ml of the chloroform was mixed with 50ml of ethanol.

Isolation of plasma

12ml of blood was collected from the vein of a healthy volunteer and was mixed with 0.5ml (500µl) of 0.5M sodium edetate to prevent it's clotting. 1ml of each concentration of (13.33µM, 26.67µM, 40.00 µM, 53.67µM and 66.67µM) was mixed separately with 1ml of blood. Thus in this way we prepared 5 test tubes. Final concentrations of $HgCl_2$ were 6.67µM, 13.33µM, 20.00µM, 26.67µM, 33.33µM respectively in these test tubes. Each of these samples was centrifuged for 5 minutes at 10,000 rpm. 0.8ml of the supernatant (plasma) was collected carefully from each of these mixtures after centrifugation and kept on ice in a sample tube till use. The remaining packed cells volume was used further for the isolation of Cytosolic Fraction.

For plasma control, 1ml of blood was mixed with 1ml of normal saline, centrifuged and plasma was collected.

Isolation of cytosolic fraction of blood

Each of the packed cell volume was washed two times with normal saline and was lysed with an equal volume of distilled water for 1 hour at 4°C. Then 0.8ml of cold chloroform:ethanol mixture was added to each of these lysed cells volume, so as to precipitate hemoglobin. Then 0.3ml of distilled water was added to each of them and the resulting mixtures were centrifuged for 5 minutes at 10,000 rpm. After centrifugation, the supernatant pale yellow (Cytosolic fraction) was collected carefully and stored on ice in a sample tube, till use.

The packed cell volume from previously centrifuged sample for isolation of plasma control was further processed for collection of Cytosolic fraction control. The Cytosolic Fraction was collected by the mentioned procedure.

Determination of biochemical inorganic parameters

All the estimations of glutathione were carried out following the modified standard Ellman's method (Ellmans, 1959) as follows:

0.2ml of each of the sample (Plasma or Cytosolic Fraction of blood) was mixed with 2.3ml buffer and 0.5ml of DTNB. Each of these mixtures was transferred to spectrophotometric cell one by one. The reference cell contained buffer. All the measurements were taken at 412nm, by using a UV-visible spectrophotometer model 1601 (Schimadzu). A DTNB blank, containing 0.5ml of 1mM DTNB and 2.5ml of buffer was also run against a reference cell containing buffer, at 412nm.

The concentration of glutathione in each sample was using the standard curve

GSH standard curve

Ellman's method was used for the construction of GSH standard curve as given in the fig. 1.

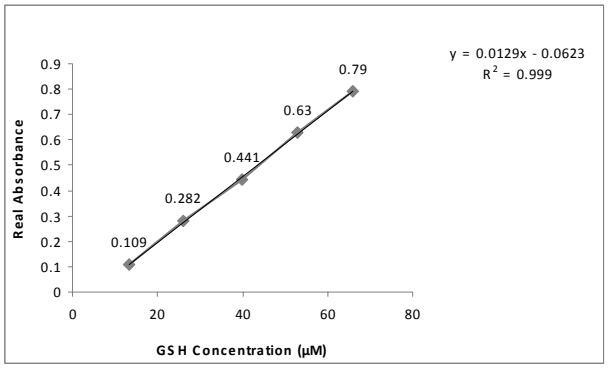

Fig. 1: Standard curve of GSH

Table 1: Effect of different concentrations of mercuric chloride on the chemical status of Glutathione (GSH) in plasma

Absorbance of 5,5-Dithiobis,2-Nitrobenzoic Acid (DTNB) blank solution was 0.060 abs. at 412nm								
S. No.	Conc. used of $HgCl_2$	Final conc. of $HgCl_2$ in mixture	1st ABS	2nd ABS	3rd ABS	Average of 3 readings	Real absorbance*	Real absorbance for plasma GSH control
1	13.33µM	6.67µM	0.305	0.293	0.311	0.303	0.250	0.484
2	26.67µM	13.33µM	0.256	0.260	0.270	0.262	0.209	0.474
3	40.00 µM	20.00µM	0.235	0.223	0.241	0.233	0.180	0.463
4	53.67µM	26.67µM	0.209	0.197	0.215	0.207	0.154	0.463
5	66.67µM	33.33µM	0.155	0.143	0.161	0.153	0.100	0.472

* Real Absorbance = Absorbance of Mixture - Absorbance of DTNB blank solution.

Table 2: Effect of mercuric chloride on the chemical status of Glutathione (GSH) in plasma with time

Absorbance of 5,5-Dithiobis,2-Nitrobenzoic Acid (DTNB) blank solution was 0.060 abs. at 412nm								
Final concentration of mercuric chloride was 33.33µM in final mixture								
S. No.	Time interval	1st ABS	2nd ABS	3rd ABS	Average of 3 readings	Real absorbance*	GSH blank ABS	Real absorbance for plasma GSH control
1	0 min	0.155	0.143	0.160	0.153	0.095	0.550	0.492
2	30 min	0.135	0.123	0.140	0.133	0.075	0.540	0.482
3	60 min	0.120	0.108	0.125	0.118	0.060	0.545	0.487
4	90 min	0.110	0.098	0.115	0.108	0.050	0.538	0.480
5	120 min	0.098	0.086	0.103	0.096	0.038	0.543	0.485
6	150 min	0.085	0.073	0.090	0.083	0.025	0.530	0.472

***** Real Absorbance = Absorbance of Mixture - Absorbance of DTNB blank solution

Effect of mercuric chloride on the extracellular plasma GSH content

Each of the plasma sample (collected by the isolation process as described above), containing increasing concentrations of mercuric chloride in the range of 6.67µM, 13.33µM, 20.00µM, 26.67µM, 33.33µM was investigated for GSH content through UV-visible spectrophotometer following the already described Ellman's method (Ellmans, 1959). There was observed that a decrease in the GSH concentration was caused by the $HgCl_2$, which was gradually increasing with increasing concentration of the $HgCl_2$ as shown in table 1 and fig.2.

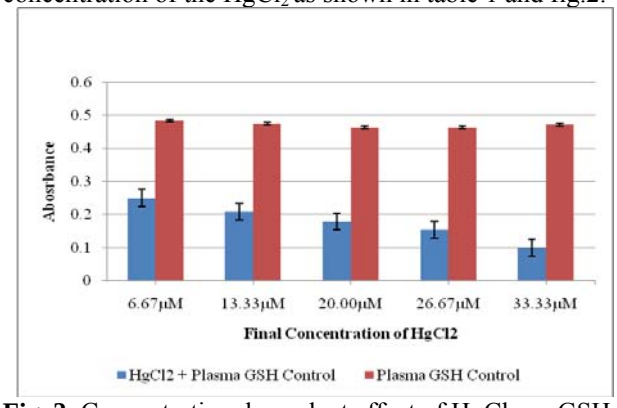

Fig. 2: Concentration dependent effect of $HgCl_2$ on GSH level in plasma.

A significant change was observed in the plasma GSH content from the control at $P<0.5$.

For time-dependent study, the GSH content was measured (by the described procedure) in sample, containing 33.33µM of $HgCl_2$ at time intervals 0-150 minutes. Again a decrease was found in plasma GSH content with time as shown in table 2 and fig. 3.

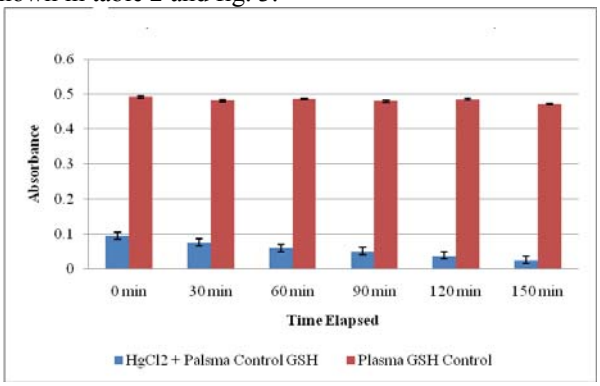

Fig. 3: Time dependent effect of $HgCl_2$ on GSH level in plasma.

Effect of mercuric chloride on the intracellular cytosolic fraction GSH content

Each of the collected cytosolic fraction sample, containing different concentrations of $HgCl_2$ (6.67µM,

Table 3: Effect of different concentrations of mercuric chloride on the chemical status of Glutathione (GSH) in cytosolic fraction.

Absorbance of 5,5-Dithiobis,2-Nitrobenzoic Acid (DTNB) blank solution was 0.060 abs. at 412nm							
Conc. used of $HgCl_2$	Final Conc. of $HgCl_2$ in mixture	1st ABS	2nd ABS	3rd ABS	Average of 3 readings	Real absorbance*	Real absorbance for CF GSH control
13.33µM	6.67µM	0.127	0.130	0.126	0.128	0.070	0.098
26.67µM	13.33µM	0.104	0.108	0.106	0.106	0.048	0.100
40.00 µM	20.00µM	0.102	0.105	0.103	0.103	0.045	0.089
53.67µM	26.67µM	0.085	0.086	0.083	0.085	0.027	0.101
66.67µM	33.33µM	0.074	0.077	0.079	0.076	0.016	0.094

* Real Absorbance = Absorbance of Mixture - Absorbance of DTNB blank solution.

Table 4: Effect of mercuric chloride on the chemical status of Glutathione (GSH) in cytosolic fraction with time.

Absorbance of 5,5-Dithiobis,2-Nitrobenzoic Acid (DTNB) blank solution was 0.060 abs. at 412nm								
Final concentration of mercuric chloride was 33.33µM in final mixture								
S. No.	Time interval	1st ABS	2nd ABS	3rd ABS	Average of 3 readings	Real absorbance*	GSH blank ABS	Real absorbance for GSH GSH control
1	0 min	0.076	0.077	0.075	0.076	0.016	0.179	0.121
2	30 min	0.074	0.074	0.074	0.074	0.014	0.176	0.118
3	60 min	0.072	0.070	0.071	0.071	0.011	0.174	0.116
4	90 min	0.069	0.070	0.068	0.069	0.009	0.173	0.115
5	120 min	0.067	0.067	0.067	0.067	0.007	0.170	0.112
6	150 min	0.064	0.065	0.063	0.064	0.004	0.167	0.109

* Real Absorbance = Absorbance of Mixture - Absorbance of DTNB blank solution.

13.33µM, 20.00µM, 26.67µM, 33.33µM) was investigated for GSH content through UV-visible spectrophotometer following the Ellman's method (Ellmans, 1959).

We found that $HgCl_2$ has caused a slight decrease in the GSH concentration. This decrease was gradually increasing with increasing concentration of the $HgCl_2$ as shown in table 3 and fig. 4.

A significant change was observed in the cytosolic fraction GSH content from the control at $P<0.5$.

For to know the time-dependent effect of barium chloride on the GSH content in cytosolic fraction, the content of GSH was measured in sample, containing 33.33µM of $HgCl_2$ at time intervals 0-150 minutes. Again a decrease was found in cytosolic fraction GSH content with time as shown in table 4 and fig. 5.

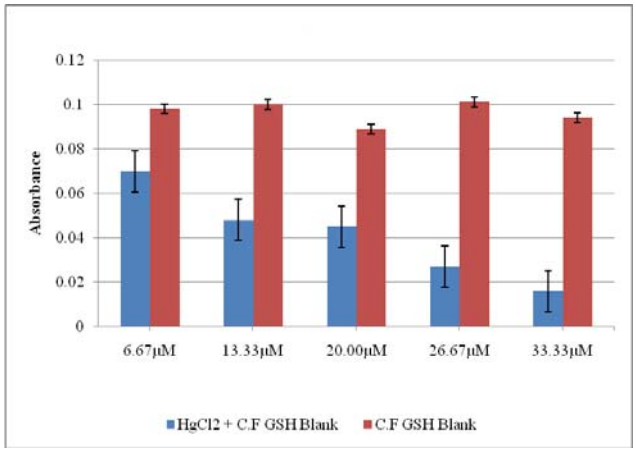

Fig. 4: Concentration dependent effect of $HgCl_2$ on C.F. GSH Level.

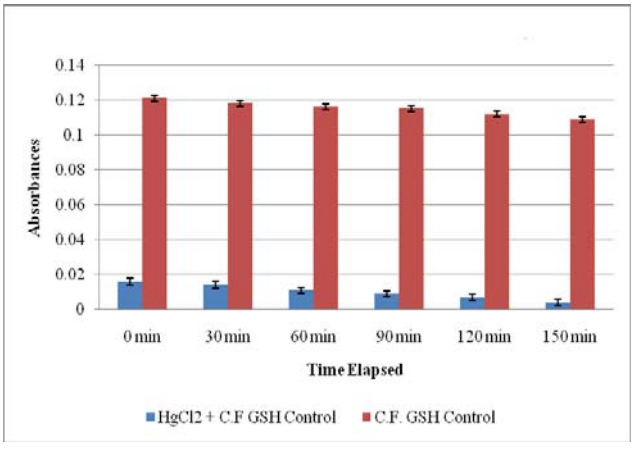

Fig. 5: Time dependent effect of $HgCl_2$ on C.F. GSH level.

DISCUSSION

In the present study our main interest was to see the effect of mercury on the important bio-molecules like glutathione *in vitro* as a model of *in vivo* reaction, to depict the picture of its acute toxicity. We have studied the complexation of the mercury to GSH in human venous blood components by UV-Visible spectroscopy. The concentrations of mercury that were used during experiments range of 6.67µM, 13.33µM, 20.00µM, 26.67µM, 33.33µM. The effect of mercury on the chemical status of Glutathione (GSH) was studied in terms of determination of concentration of GSH at λ_{max} 412nm. This λ_{max} (412nm) is being used for the determination of GSH concentration especially thiols in samples by a well-known Elman's method. According to our findings mercury induced the depletion of GSH in blood components. Our results may confirm the relationship between mercury mediated toxicity and decreased GSH levels. Our results conform with early findings, particularly central nervous system that excessive MeHg ingestion from a diet rich in fish has been linked to aberrant central nervous system (CNS) function. This ubiquitous environmental contaminant is capable of causing toxic effects as indicated by human poisoning epidemics following food-borne MeHg ingestion (Bakir *et al*., 1973; Takeuchi, 1972). MeHg can easily pass placental and blood–brain barriers and cause CNS damage to both adult and developing brain (Clarkson, 1997; Lepham *et al*., 1995). Maternal mercury ingestion during pregnancy causes neurological as well as neuropsychological deficits in the offspring (Cordier *et al.,* 2002; Grandjean *et al*., 2003). The exact mechanism of the neurological and neuropsychological deficits caused by MeHg still needs to be fully investigated.

One of the postulated mechanisms of MeHg-associated neurotoxicity is enhanced glutamate release (Aschner *et al*., 1993) through the mediation of free radical species like INO or H_2O_2 (Allen *et al*., 2001: Bal-Price *et al*., 2002). Increased levels of glutamate in extracellular space are not only toxic to astrocytes but can also stimulate neuronal N-methyl-daspartate (NMDA) receptors of juxtaposed neurons leading to neurotoxicity (Aschner, 2000; Bal-Price *et al*., 2002). Depleted GSH levels following MeHg exposure may also contribute to neurotoxic damage) (Bolanos *et al*., 1996). Several toxic reactive species (i.e., IO2 _, INO, and ONOO_) have been found to be damaging to the activities of various mitochondrial complexes in GSH-depleted astrocytes or in neurons with relative scarcity of GSH (Bolanos, *et al*., 1996; Stewart *et al*., 2002).

There is a possibility of decreased level of GSH contact which may be due to the Hg-SH complex formation or there may be oxidation of reduced glutathione (GSH) to oxidized glutathione GSSG. These results for $HgCl_2$ may conform with our previous findings (Khan *et al*., 2010; Khan *et al*., 2009; Khan *et al*., 2008), GSH level in all of these studies was decreased in the same manner.

$$GSH + HgCl_2 \rightarrow Hg\text{-}GS \text{ complex}$$

This research work showed that metalloelement like Hg has concentration and time wise effect on decreasing the concentration of GSH in plasma and cytosolic fraction. Glutathione has a reducing capacity for exogenous compounds like mercury and converts itself to oxidized state which is a disulphide (GSSG).

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