ORIGINAL ARTICLE

EFFECT OF MERCURY ON REDUCED GLUTATHIONE OF HUMAN T AND B LYMPHOCYTES: A COMPARISON OF INORGANIC AND ORGANIC MERCURY

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ABSTRACT

Background: Environmental pollution of transition metals especially of mercury is of great concern to the researchers in medical field since long. Mercury causes oxidative stress in all the cells including lymphocytes of the human immune system. Effect of inorganic compound of mercury was compare with our own report of effect of organic compound of mercury on T and B lymphocytes in human blood.

Material & Methods: The present experimental work was design to analyze the concentration and time dependent effect of mercuric chloride on the chemical modulation of T and B-lymphocytes GSH of human blood. Terasaki technique for lymphocytes isolation and then subsequently separation of T and B-cells was used. Quantification of thiol in T and B-cells was done by Ellman's method.

Results: The lowest used concentration of mercuric chloride depleted T and B-cells GSH level 2.115 μM (27.34%) and 2.019 μM (32.41%) respectively which was significant ($p<0.001$) decrease in both T and B-cells GSH level. This effect of mercury was dose and time dependent.

Conclusion: This study suggests that depletion of GSH due to inorganic mercury in T and B-lymphocytes is more severe as compared to its organic form.

KEY WORDS: Antioxidant; Mercuric chloride; T-lymphocytes; B-lymphocytes; Pollutants; Lipid peroxidation.

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INTRODUCTION

Glutathione is the major antioxidant of low molecular weight containing –SH group present in mammalian cells, in millimolar amounts in most cells.[1] All forms of mercury regardless physical or chemical form are one of the most dangerous pollutants in environment.[2-4] There are evidences that mercury reacts with reduced form of glutathione due to which in many specific types of cells glutathione contents are decreased as in human erythrocytes[5,6] indicating that GSH is the big oxidant protectant. Mercury (Hg) is highly volatile substance which can easily be absorbed across the biological membranes where Hg accumulation and attachment to tissues for long term is established while mercury also causes production of extremely toxic hydroxyl radicals thus further depleting glutathione.[7]

GSH depletion can enhance neurological damage as for example in Parkinson's disease.[8] Thiol group of glutathione becomes attached with mercury resulting in preventing mercury from being binding to cellular proteins thus GSH provides safe guard against damage to enzymes as well as tissues.[9] Complexes of GSH with Hg in liver, kidney, and brain are found showing the primary route of transported and elimination of Hg from the body.[7] Similarly complexes of GSH and Hg are also found in bile and urine.[10] Glutathione manages the rate of mercury efflux in bile as there is no effect of increase or decrease of bile flow rate on mercury efflux because mercury efflux is inversely proportional to bile flow rate thus net mercury efflux remains unchanged[11,12] and this fact is practically established in several animals.[13-15] In brain astrocytes are the cells which act as primary

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defense against all toxic materials and these are the cells were Hg accumulate.[16] Astrocytes prevent all metals from crossing the blood-brain barrier as these cells are rich of glutathione contents and are considered to be the big depot of Hg.[17] Addition of glutathione and its precursors considerably increases the discharge of mercury from astrocytes cells so conjugation of GSH with Hg is the main route of mercury elimination from these cells.[18] Several studies have shown that GSH increases Hg amputation from renal tissue for example in mammalian renal cells GSH is fifty percent more effective as a chelating agent than 2,3-dimercaptosuccinic acid (DMSA) in preventing inorganic mercury buildup in renal cells.[19] Mercury produces singlet oxygen, hydrogen peroxides etc against which GSH increases the antioxidant capacity of the cell thus providing a defense mechanism.[9] Experimentally it was proved that addition of glutathione to cell cultures that was exposed to mercury has prevented the decrease of cellular levels of glutathione peroxidase.[9] GSH provides protection against renal damage due inorganic mercury toxicity. In an experiment of incubation of rat renal cells with reduced glutathione and mercuric chloride was drastically more protective of renal cell injury as compared to inorganic mercury exposure alone.[20] GSH, vitamin E, and ascorbic acid are exhausted in renal tissue exposed to inorganic mercury ($HgCl_2$) while addition of GSH increased levels of both vitamin E and ascorbic acid in renal cells exposed to $HgCl_2$.[7] Stability constants are very high for mercury and glutathione so Hg^{II} binds to GSH freely which is in the highest concentrations in cells.[21]

The aim of this study was to evaluate the effect of mercury on human T and B lymphocytes GSH by using Terasaki technique of T and B-lymphocytes separation and Ellman's method of thiol quantification.

MATERIAL AND METHODS

Terasaki technique was used to segregate components of WBC's which is a density gradient separation technique usually carried out with suitable medium. This medium separates the components of WBC's density wise through centrifugation process as mentioned below.

In heparinized tube, 10 ml fresh venous blood from healthy human volunteer was taken. Then 4ml of Histopaque was added in two separate test tubes. To these two tubes 4ml of venous blood slowly and carefully was added. (As blood should not disturb Histopaque layer below and it should form separate layer over Histopaque) and these two tubes were centrifuged at 1800 rpm for 35 minutes. As a result, 5 discrete layers from top to battom were Plasma, lymphocytes, Histopaque, Neutrophils and RBC's were produced .The top most layer of plasma was removed in separate test tube with the help of a micro-pipette for further use. Lymphocytes layer and also half of Histopaque layer was collected in another centrifuge tubes while to each of these tubes enough of RPMI was added to makeup the final volume and were centrifuged at 1800 rpm for 15 minutes. Upper layer was discarded while Lymphocytes were sedimented at the base of test tubes. The sedimented lymphocytes were resuspended by adding 5 ml of RPMI in each test tube. At this moment 3 ml of 20% sucrose was added to the base of each test tube. Sucrose was added to eradicate the platelets from the lymphocytes. Then it was centrifuged at 700 rpm for 15 minutes. Lymphocytes were sedimented and the upper layer was discarded.

Then 0.5-1 ml RPMI was added to the clump of lymphocytes in each test tube. 0.5 ml double refined distilled water was further added to these tubes for the purpose to lyse RBC's if any by slightly shaking the tubes. Again 5-10 ml of RPMI was added to both the test tubes making final volume of tubes equal. These test tubes were centrifuged at 700 rpm for 5 minutes. Above the lymphocytes clump upper layer was discarded. The sedimented lymphocytes were resuspended with 1-2ml of RPMI. Now at this stage lymphocytes were separated.

The above separated lymphocytes were now further isolated into its types T-lymphocytes and B-lymphocytes.For separation of T-lymphocytes, the column (using 1 ml syringe) was prepared upright containing nylon wool; it was ensured that nylon wool has already been incubated at 37^0C for 30 minutes. The Lymphocytes sample was poured in the column slowly.

We waited till we saw that few drops from the column have been dropped in the test tube below. Now we have added slowly 10ml of RPMI to the column.Drops from the column containing T-lymphocytes were poured in the test tube. Now this test tube contained components of Lymphocytes i.e. T-Cells. (T-Cells have smooth, outline, round shape and do not stuck to the nylon wool.

Now we added 5-10ml RPMI slowly to the above mentioned column where B-lymphocytes have stuck to the nylon wool because of their irregular shape. Now we squeezed the nylon wool slowly with glass rod in the column. Drops were started falling in the tube, (new tube), the squeezing process was completed and we ensured that all drops from the column have been collected in the tube. These collected drops contained the B-lymphocytes. 50 μl HCl (0.1N) was now added to this tube. It was sealed tightly and placed on ice or freezer/Refrigerator till further use. Following chemicals and apparatus were used:

RPMI-1640, Fetal calf serum, (>98%; agarose gel electrophoresis lyophilized), Potassium dihydrogen phosphate (Merck), Reduced glutathione (Fluka), Ficol paque(Sigma Aldrich),NaH_2PO_4 (Merk),10M Perchloric Acid 70% (fluka),NaCl (Merck), Chloroform , Ethanol (Merck), Hydrochloric acid, HCl 35% (Kolchlight),pH-Tablets (pH; 4 & 7), NaCl (Fluka), Sodium edetate (Riedel Dehean AG Sleeze Hannover), Dextrose (Merck), Micropipettes of various capacities (Socorex , Finland), U.V-visible spectrophotometer (Schimadzu, 1601 Japan), Korean pH meter (210-NOV),Hot plate-400 (UK), Magnetic Stirrer, Eppendolf's tubes (Plastic, 10l), Potter-eveljhem homogenizer (japan), Centrifuge (H-200,Kokusan Ensink company Japan), Sterile pyrogen free disposable syringes (Surge Pharmaceuticals), Analytical weighing balance AX 200 (Schimadzu, Japan), Siliconized glass test tubes, Chromatographic column.

RESULTS

Interaction between six different concentrations of mercuric chloride with GSH of T and B-cells (isolated) was evaluated. Absorbance of each sample mixture was recorded under UV-visible spectrophotometer at fixed wave length λmax: 412 nm and then each absorbance was converted to concentration of T-cells/ B-cells GSH. This concentration of unknown T-cells/B-cells GSH left after the interaction of various concentrations of mercuric chloride with T-cells/B-cells GSH was calculated by using standard curve for known concentration of GSH and was found decrease in T-cells/ B-cells GSH level significantly ($p<0.001$). Depletion in GSH contents in all the six sample mixtures (T-cells + different concentrations of mercuric chloride, B-cells and different concen-

Table 1: Result of various concentrations of mercuric chloride (MC) on the modulation and chemical status of T-lymphocytes (After separation).

	Parameters	**0.003**			**0.03**	**0.33**	**3.33**	**33.33**	**66.66**
	Time (Minutes)	Conc:			Conc:	Conc:	Conc:	Conc:	Conc:
1	Remaining concentration of GSH at 0 mint	2.115			2.025	1.943	1.752	1.665	1.611
	pH		7.0	2.280	2.172	2.096	1.981	1.930	1.834
			7.5	1.796	1.713	1.624	1.529	1.452	1.363
			8.0	1.949	1.866	1.783	1.682	1.611	1.522
			8.5	2.089	2.000	1.924	1.828	1.752	1.662
	Temperature (°C)		25	2.312	2.210	2.146	2.019	1.930	1.854
			37	2.172	2.096	2.006	1.847	1.834	1.745
			45	2.255	2.159	2.108	1.987	1.924	1.854
2	Remaining concentration of GSH at 20 mint	1.834			1.656	1.573	1.395	1.338	1.242
3	Remaining concentration of GSH at 40 mint	1.599			1.484	1.401	1.217	1.166	1.083
4	Remaining concentration of GSH at 60 mint	1.420			1.344	1.268	1.134	1.032	0.955
5	Remaining concentration of GSH at 90 mint	1.306			1.217	1.108	1.025	0.987	0.885
6	Remaining concentration of GSH at 120 min	1.223			1.153	1.083	0.968	0.898	0.815
	T-lymphocytes GSH Ctrl	2.911			2.911	2.911	2.911	2.911	2.911

Values are given as Means±S.E.M. n=3. Significance of differences among the various groups was evaluated by one-way ANOVA followed by Dunnet's HSD test. $p<0.001$ vs. control (T-cells GSH)

Table 2: Result of various concentrations of mercuric chloride (MC) on the modulation and chemical status of B-lymphocytes with time (After separation).

	Parameters	**0.003**			**0.03**	**0.33**	**3.33**	**33.33**	**66.66**
	Time (Minutes)	Conc:			Conc:	Conc:	Conc:	Conc:	Conc:
1	Remaining concentration of GSH at 0 mint	2.019			1.917	1.650	1.478	1.408	1.357
	pH		7.0	1.803	1.707	1.669	1.586	1.554	1.497
			7.5	1.312	1.248	1.197	1.134	1.076	0.962
			8.0	1.471	1.408	1.357	1.293	1.236	1.185
			8.5	1.618	1.541	1.497	1.427	1.382	1.325
	Temperature (°C)		25	1.828	1.745	1.720	1.624	1.554	1.516
			37	1.694	1.631	1.580	1.516	1.516	1.408
			45	1.771	1.701	1.675	1.592	1.548	1.510
2	Remaining concentration of GSH at 20 mint	1.809			1.637	1.541	1.350	1.306	1.217
3	Remaining concentration of GSH at 40 mint	1.573			1.452	1.357	1.191	1.140	1.064
4	Remaining concentration of GSH at 60 mint	1.382			1.299	1.223	1.108	1.000	0.924
5	Remaining concentration of GSH at 90 mint	1.261			1.178	1.127	1.000	0.955	0.860
6	Remaining concentration of GSH at 120 min	1.191			1.115	1.057	0.924	0.860	0.777
	B-lymphocytes GSH Ctrl	2.987			2.987	2.987	2.987	2.987	2.987

Values are given as means ± S.E.M. n=3. Significance of differences among the various groups was evaluated by one-way ANOVA followed by Dunnet's HSD test. p 0.001 vs. control (B-cells GSH).

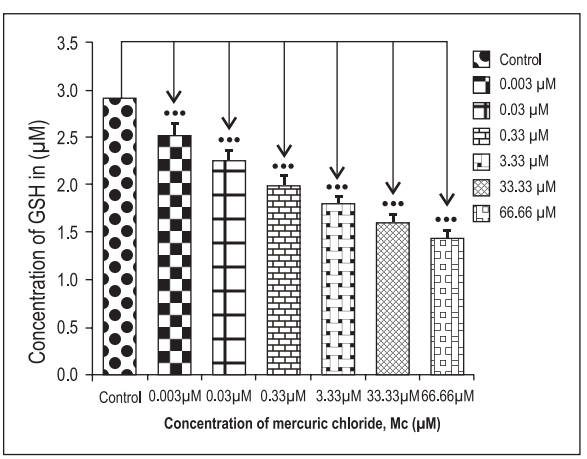

Figure 1: Effect of different concentrations of mercuric chloride (MC) on the chemical status of T-lymphocytes-GSH level (concentration effect). Results are the mean ±S.E.M. of 3 experiments of T-lymphocytes fraction. Significance of differences among the various groups was evaluated by One-way ANOVA followed by Dunnet's HSD test. ***p 0.001vs. control (T-cells GSH).

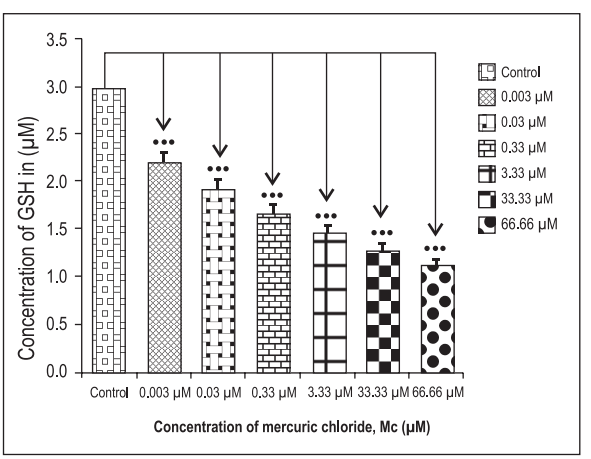

Figure 2: Effect of different concentrations of mercuric chloride (MC) on the chemical status of B-lymphocytes-GSH level (concentration effect).Results are the mean ±S.E.M. of 3 experiments of B-lymphocytes fraction. Significance of differences among the various groups was evaluated by One-way ANOVA followed by Dunnet's HSD test. *** p 0.001vs. control (B-cells GSH).

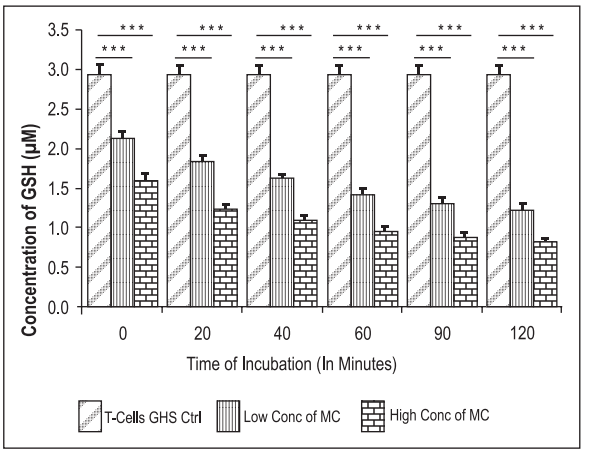

Figure 3: Effect of mercuric chloride (MC) on T-lymphocytes-GSH with time of incubation from 0 to 120 min: Results are the mean ± S.E.M of 3 experiments of T-lymphocytes-GSH. Significance of differences among the various groups was evaluated by One-way ANOVA followed by Dunnet's HSD test. *** p 0.001 vs.control (T-cells GSH).

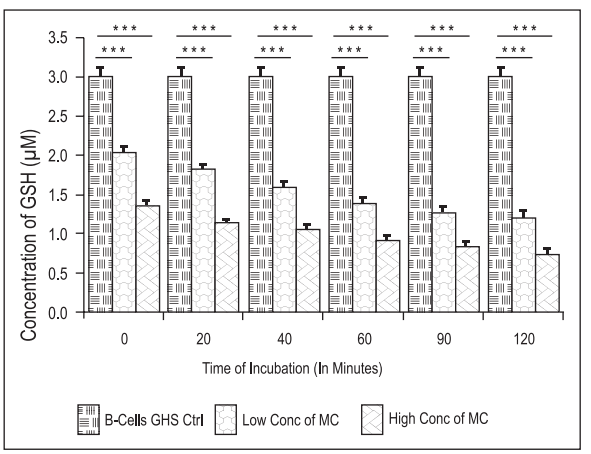

Figure 4: Effect of mercuric chloride (MC) on B-lymphocytes-GSH with time of incubation from 0 to 120 min: Results are the mean ± S.E.M of 3 experiments of B-lymphocytes-GSH. Significance of differences among the various groups was evaluated by One-way ANOVA followed by Dunnet's HSD test. *** p 0.001 vs.control (B-cells GSH).

trations of mercuric chloride) was inversely proportional to the metal concentrations, with the increase in metals concentration there was decrease in GSH contents accordingly. The drop in GSH contents of T-cells by all the used concentrations was up to 2.115μM(27.34%),2.025 μM (30.44%), 1.943 μM (33.25%), 1.752 μM (39.81%),1.665μM (42.80%) and 1.611 μM (44.66%) respectively with respect to T-cells GSH control (Fig. 1) while the decrease in GSH level of B-cells GSH was 2.019 μM (32.41%), 1.917 μM (35.82%), 1.650 μM (44.76%), 1.478 μM (50.52%), 1.408 μM (52.86%) and 1.357μM (54.57%) by all the six used concentrations of mercuric chloride with respect to B-cells GSH control. (Fig. 2) The decrease in B-cells GSH level is greater than decrease in T-cells GSH level showing that mercuric chloride has the penetrating capability into the semipermeable membrane of B-cells of human blood.

Drop in GSH level of T-cells and B-cells with the passage of time was also investigated during the experiment of interaction between various concentrations of mercuric chloride with T and B-cells GSH contents by incubating these different concentrations of mercuric chloride with T and B-cells GSH at different time of incubation while these incubation times were 0, 20,40,60,90 and 120 minutes. It was observed that with the increase in incubation time between various concentrations of mercuric chloride and T-cells/ B-cells GSH, there is further significant ($p<0.001$) drop in GSH level of T and B-cells. In case of T-cells GSH content (Table 1) the drop in T-cells GSH contents from 0 to 120 minutes was 1.223μM (42.01%), 1.153 μM (39.60%), 1.083μM (37.20%), 0.968 μM (33.25%), 0.898 μM (30.85%) and 0.815 μM (27.99%) by all the used concentrations of mercuric chloride with respect to T-cells GSH control while in case of B-cells (Table 2) the decrease in B-cells GSH level by all used concentrations of mercuric chloride was 1.191 μM (39.87%), 1.115 μM (37.33%), 1.057 μM (35.39%), 0.924 μM (30.93%), 0.860μM (28.79%) and 0.777μM (26.01%) with respect to B-cells GSH control. The effect of lowest and highest used concentration of mercuric chloride on T and B-cells GSH (Figure 3,4) indicate that with the passage of time there is further significant drop in GSH level of T and B-cells.

DISCUSSION

Toxic metals like cadmium, mercury have high affinity for sulfhydryl group thus –SH group of GSH binds directly to these toxic heavy metals. Our results provide an insight into the role of glutathione in metal induces toxicity especially of mercury metal while our findings also provide evidence that this metal has high chemical affinity for –SH group present in non-protein glutathione and thus is capable of interacting nuclear proteins and DNA as a result of which site specific damages occur. Moreover this study shows that in-organic form of mercury causes greater drop in T and B-lymphocytes GSH as compare to organic form of mercury reported by us in our previous study.[22] We suggest that the possible reason for this is that inorganic compounds dissociate more into negative and positive ions than organic compounds so more availability of mercuric ions increase the chances of depletion of GSH more. Hg^{+2} ions have high affinity to bond with reduced sulfur atoms especially those present on the endogenous thiol-containing molecules like glutathione.

It is suggested that depletion of GSH was probably due to $Hg(GS)_2$ adduct formation or GSSG formation. As a result of the binding of Hg^{II} to glutathione and the subsequent elimination of intracellular glutathione, levels of GSH are lowered in several specific types of cells on exposure to all forms of mercury. Some of the specific changes that lead to tissue damage and death in chronic exposure have been related to oxidative stress and thiol depletion. Cellular damage results from Hg^{II} binding to sulfhydryl groups in tissue, the production of lipid peroxides, and the depletion of glutathione. Hg^{II} also has a very high affinity for glutathione and can form a complex with glutathione that is eliminated in bile. Interaction of glutathione and xenobiotics is necessary facet of physiological metabolism which results in depletion of GSH and is used as tool to study the role of GSH in antioxidant defense. In our present study the drop in T-cells GSH level by lowest used concentration of mercuric chloride was 2.115μM (27.34%) and in B-cells GSH level was 2.019 μM (32.41%), while by lowest used concentration of phenyl mercuric acetate drop of T and B-cells GSH was 2.306μM(23.28%) and 2.108μM (30.61%) respectively similar difference in T and B –cells GSH depletion is found by other used concentrations of mercuric chloride for example highest used concentration of mercuric chloride has depleted T and B-cells GSH up to 1.611 μM (44.66%) and 1.357μM (54.57%) in T and B-cells while highest used concentration of phenyl mercuric acetate drop T and B-cells GSH 1.783μM(59.31 %) and 1.414 μM (46.54%) respectively which clearly indicates that inorganic form of mercury cause greater depletion than organic form of mercury in T and B-cells GSH[22,23] and also shows that mercury has greater penetrating capability into the semipermeable membrane of B-cells of human blood. In T and B-cells, interaction of Hg^{II} with GSH provides concentration dependent protection from Hg-induced cytotoxicity as complex of Hg with GSH limits and regulates its reactivity as well as facilitating its transportation and removal from the cell. Although several studies attempted to explicate the mechanisms concerned in mercury toxicity but still research is required to get better pharmacological action. The only drugs available nowadays to limit metal toxicity are chelating agents but their use is often limited by their lack of selectivity as they cause elimination of essential metal ions, so making it imperative to identify novel natural substituents that permit the elimination of toxic metals like mercury from the body without disturbing physiological ionic homeostasis.

CONCLUSION

Glutathione and xenobiotics including heavy metals interaction is necessary facet of physiological metabolism which results in depletion of GSH and is used as tool to study the role of GSH in antioxidant defense and hence patients suffering from metals toxicity especially due to inorganic compounds of metals should also be given suitable antioxidants or their precursors as such patients have weak immune system. Improvement in immune system of patients of various diseases and especially in patients of metals toxicities will save them from other complications. This study suggests that depletion of GSH due to inorganic form of mercury in T and B lymphocytes is more severe as compared to its organic form reported previously.

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CONFLICT OF INTEREST
Authors declare no conflict of interest.
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None declared.