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In vitro Study Involving the Comparative Effect of Heavy Metal Ions on Antioxidant Enzymes Activity and Lipid Peroxide Levels in Human Erythrocytes

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Abstract: The effect of the concentration rise in Cu, Zn, Pb and Cr salts (10-100 μM) on lipid peroxidation (MDA) and the activities of peroxide metabolism enzymes: catalase, glutathione peroxidase, reductase and glutathione s-transferases were investigated in human erythrocytes. Copper and lead were found to have more pronounced effect than zinc and chrome on the erythrocytes cell membrane phospholipid oxidation and MDA formation. In human erythrocytes, Cr significantly increased the glutathione peroxidase (GPx) and decreased the Glutathione Reductase (GR), while no significant difference ($p<0.05$) in glutathione s-transferase (GST) and catalase (CAT) activities at 100 μM were observed. Zinc increased the GPx and CAT and decreased the GR, whereas GST activities do not changed ($p<0.05$). Lead significantly increased GPx and GST and no effect on GR and CAT activities was observed ($p<0.05$). However, copper showed no significant effect on GPx and GST activities, but significantly caused increased in CAT and a decrease in GR activity ($p<0.001$). These data show that, heavy metal ions had only little effect on haemolysis and statistical variable effects on antioxidant enzyme activities in human erythrocytes and lipid peroxide level when compared to control values. It was concluded that metal ions produced variable toxic effects on human erythrocytes, so that the mechanism Cu and Pb-induced membrane lipid oxidation may be different from that caused by Zn and Cr ions.

Key words: Antioxidant enzyme, erythrocyte, heavy metals, lipid peroxide

INTRODUCTION

Redox active transition metals such as copper (Cu) and zinc (Zn) are useful to cell as well as they can be dangerous trace elements. Indeed, trace element is essential since it acts as cofactor of many enzymes. However, its redox reactivity leads to risk of damage to cell and tissues. Reaction of these elements with oxygen produces very reactive and potentially toxic oxygen species (Pena *et al*., 1999).

Both occupational and environmental exposures to hazardous heavy metals, such as chromium (Cr) and lead (Pb) posses significant toxicological concerns. Not only do these metals lead to acute toxicity at higher concentrations, but they may also mediate the development of additional pathologic conditions in individuals exposed chronically to low levels. Environmentally relevant metals seldom occur alone, rather, they most often occur in hazardous waste sites or ground water supplies in combination with other contaminants. This project is, therefore, an attempt to test such an additively concept at low exposure levels. The metals chosen for our studies are highly relevant to human exposure. Cd, Cr and Pb are the top metals in site frequency count by the ATSDR Completed Exposure Pathway Site Count Report (Johnson and DeRosa, 1995, 1997); of these, As, Pb and Cd are among the Superfund Top 10 Priority Hazardous Substances (DeRosa and Johnson, 1996), i.e., those considered posing the greatest hazard to human health.

The red blood cells are particularly exposed to damage induced by Reactive Oxygen Species (ROS) because of high content of substrates easily oxidizable, such as membrane polyunsaturated lipids and influx of oxygen. Blood is the best indicator of the internal exposure to heavy metals. The erythrocytes is the main target for heavy metals toxicity that lead to inhibition of heme biosynthesis enzymes, hemolytic and peroxidation of red blood cells membranes (Battistinin *et al*., 1971; Ribarov *et al*., 1981; Hsu, 1981; Clemens and Waller, 1987; Sies, 1993; Delatycki M *et al*., 1999, Lanphear *et al*., 1999; Michiels *et al*., 1994; Fridovich, 1997). The protection of cells against heavy metals and oxidative stress, generally includes the detoxication effect mediated by the antioxidant system including the glutathione and glutathione-detoxication enzyme (Sies, 1993; Michiels *et al*., 1994, Fridovich, 1997). Moreover, heavy metals at high doses might attenuate the activities of antioxidant enzymes such as Glutathione Reductase (GR), transferases (GSTs) and peroxidase (GPx) and catalase (CAT) (Sugawara *et al*., 1991; Hunaiti *et al*., 1995; Halliwell and Gutteridge, 1984).

Most previous studies were done on industrial workers. For these reasons it was important to examine

the effect of heavy metals on red blood cells *in vitro* and relate these to red blood cells glutathione-free radical scavenging enzymes such as glutathione reductase, transferases and peroxidase as well as catalase and lipid membrane peroxidation.

MATERIALS AND METHODS

Chemicals: Lead chloride, glutathione reductase, t-butylhydroperoxide, reduced glutathione and 1-chloro-2, 4-dintrobenzene were purchased from Sigma-Aldrich Chemical Co. (Germany). Potassium dichromate was purchased from Fluke (Germany). Hydrogen peroxide was purchased from GCC (England). NADPH and oxidized glutathione were purchased from Applichem (Germany). Copper (II) chloride was purchased from Neu tech ltd (England). Zinc chloride, was obtained from SDS Fine Chemical Company (India).

Blood sample: Fresh heparinized blood samples were collected from healthy volunteers who were not occupationally exposed to heavy metals. Whole blood were separately incubated for 24 h at 37°C in the presence of 10, 50 and 100 μM of lead chloride, potassium dichromate, copper (II) chloride and zinc chloride. Control without heavy metals was included each time.

Hemoglobin measurement: The Hb concentration was determined by the cyanmethaemoglobin method (Drabkin and Austin, 1935).

Measurement of enzymes activities involved in oxidants metabolism: The activities of GST, GR, GPx and CAT enzymes were determined in lyses red blood cells after incubation with metals for 24 h using a Genesis UV/VIS-Spectrophotometer. One milliliter of 0.9% NaCl was added to 1 mL of Erythrocytes and 0.1 mL of this was taken and added to 0.1 mL of distilled water to prepare 1:20 hemolysate, which was used for antioxidant enzymes measurements.

Glutathione S-transferase (GST) assay conditions: GST activities were measured by the spectrophotometric assay of Habig *et al.* (1974). It uses 1-chloro-2,4-dinitrobenzene (CDNB) as electrophilic substrate that binds to GSH with the participation of the enzyme and forms a colored GSH-substrate complex, detected at 340 nm. Controls without CDNB were used and replaced by distilled water were subtracted from each assay. The specific activity of GSTs was expressed as μmol/min/g hemoglobin.

Glutathione reductase (GR) assay conditions: GR activity was assayed as described previously (Gupta *et al.*, 1999). Assay mixtures consisted of 83 μmol Tris (pH 8.0), 0.8 μmol of EDTA, 5.70 μmol GSSG in 0.1 M Tris (pH 7.0) and 0.2 μmol NADPH in total volumes of 980 μL. To the above mixtures, 20 μL of lyses cells were added, mixed rapidly and rates of oxidation of NADPH followed spectrophotometrically at 340 nm. Control without NADPH was used and subtracted from each assay. The specific activity was expressed as μmol/min/g hemoglobin

Glutathione peroxidase (GPx) assay conditions: GPx activity with t-butyl-hydroperoxide was determined according to the method of Lawrence and Burk (1976) Briefly, the reaction mixtures consisted of 50 mM phosphate buffer (pH 7.0), 1 mM EDTA, 0.2 mM NADPH, 1 U mL^{-1} GR, 1 mM GSH and 1.5 mM t-butyl-hydroperoxide. All ingredients except lysed cell and peroxide were combined at the beginning of each experiment. Twenty microliter of Lytic cells were added to 880 μL of the reaction mixtures and allowed to incubate for 2 min at room temperature before initiation of the reaction by the addition of 0.1 mL of peroxide solution. Absorbances at 340 nm were recorded for 3 min and the activities were expressed as μmol NADPH oxidized/min/g Hb (mU/g Hb). Blank reactions with lytic cell replaced by distilled water were subtracted from each assay.

Catalase (CAT) assay conditions: Catalase activity was measured spectrophotometrically by monitoring the decrease in A_{240} resulting from the elimination of H_2O_2 as described previously (Hildebrandt and Roots, 1975). The ϵ for H_2O_2 at 240 nm was 43.6 M^{-1} cm^{-1}. The standard reaction mixture for the assay contained 50 mM phosphate buffer (pH 7.0), 10 mM H_2O_2 and 20 μL of hemolysate for a total volume of 1.0 mL. The reaction was run at 25°C and only the initial linear rate was used to estimate the catalase activity. The amount of enzyme activity that decomposed 1 μmol of H_2O_2 per min was defined as 1 U of activity. The activity was expressed in unit per g of hemoglobin. Blank reactions with lytic cell replaced by distilled water were subtracted from each assay.

Determination of malondialdehyde: Blood MDA (mmol L^{-1}) was determined by the double heating method of Draper and Hadley (1990). Erythrocytes were lysed with distilled water prior to lipid peroxide measurement. This method was used to obtain a spectrophotometric measurement of the color produced, which is based on the reaction of the end products of lipid peroxidation with tiobarbituric acid. For this purpose, 2.5 mL of 10% (w/v)

trichloroacetic acid solution was added to 0.5 mL erythrocyte in each centrifuge tube and placed in a boiling water bath for 15 min. The mixture was cooled in tap water and centrifuged at 1000 g for 10 min. Then 2 mL of the supernatant was added to 1 mL of 0.67% (w/v) TBA solution in a test tube and placed in a boiling water bath for 15 min. The solution was cooled in tap water and its absorbance was measured with a spectrophotometer at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of the MDA-TBA complex 1.56×10^5 cm^{-1} M^{-1} and expressed in μmol g^{-1} Hb erythrocyte.

Statistical analysis: Data were expressed as means±SEM (n = 5) (results obtained from 5 different experiments). Significant differences between control and heavy metal treated cells were determined by one-way ANOVA. Differences were considered significant when $p<0.05$.

RESULTS

Range of metal ions concentration: The metal ions Cr, Cu, Pb and Zn were used at concentration ranged from 10-100 μM. Preliminary experiments were carried out to determine the concentrations causing no cell haemolysis after 24 h of incubation (data not shown).

Effect of copper: Red blood cells displayed a higher sensitivity to $CuCl_2$ than to $K_2Cr_2O_7$, since 24 h exposure to 100 μM $CuCl_2$ resulted in partially haemolysis; no haemolysis was observed with 10 to 50 μM $CuCl_2$. Similarly 10-100 μM Cu did not induce any statistically significance changes in total peroxidase activity. However, numerically, there was an increase in the GPx activity. In contrast, GR showed decrease in activity related to the Cu concentration. At 100 μM Cu, this activity was appreciably altered and the activity had decreased by 43% relative to the control. Catalase activity was significantly increased ($p<0.001$) with gradual increase of Cu. The CAT activity of cells treated with 10-100 μM Cu showed approximately five folds increase relative to the control. Whereas no significant differences between the activities of GPx, GR and GSTs ($p<0.05$) when compared to control (Table 1).

Effect of dichromate: After 24 h of incubation, human erythrocytes were not hemolysed by 100 μM potassium dichromate. At concentrations (10-100 μM), dichromate exposure had no marked effect on GSTs and CAT activities. Catalase and GSTs activities appeared to be unaffected by the lowest concentrations of dichromate (up to 100 μM). The GPx activity of cells treated with

Table 1: *In vitro* copper effects on antioxidants enzymes activities in human red blood cells after 24 h exposure

	$CuCl_2$ concentration (μM)[#]					
Enzyme	Control (0)	10	50	100	SE	Sig.
GPx (U/g Hb)	11.05	11.58	11.99	13.05	1.51	NS
GR. (mU/g Hb)	1.24	1.44	1.38	0.71	0.34	NS
GSTs (U/g Hb)	3.92	3.89	3.56	3.59	0.15	NS
CAT. (U/g Hb)	4.18	8.73^a	19.44^b	19.44^b	1.30	***

Values are mean±SE, n = 5. ***Significantly different from the control as $p<0.001$. NS: Not Significant. [#]Human erythrocytes Antioxidant enzyme (GPx, GR, GSTs and CAT) activities were measured after 24 h of exposure to increasing concentrations of $CuCl_2$

Table 2: *In vitro* chromate effects on antioxidant enzymes activities in human red blood cells after 24 h exposure

	$K_2Cr_2O_7$ concentration (μM)[#]					
Enzyme	Control (0)	10	50	100	SE	Sig.
GPx (U/g Hb)	11.05^a	7.15^b	9.87^{ab}	16.55^c	1.08	***
GR. (mU/g Hb)	1.24^a	2.88^b	5.60^c	1.44^a	0.67	**
GSTs (U/g Hb)	3.92	3.47	3.71	2.28	0.76	NS
CAT. (U/g Hb)	4.18	3.85	4.97	3.99	0.71	NS

Values are mean±SE, n = 5. ** Significantly different from the control as $p<0.01$. *** Significantly different from the control as $p<0.001$. NS: Not Significant. [#]Human erythrocytes Antioxidant enzyme (GPx, GR, GSTs and CAT) activities were measured after 24 h of exposure to increasing concentrations of $K_2Cr_2O_7$

100 μM a dichromate showed an approximately 50% increase relative to the control, whereas this activity was appreciably depressed at lower concentrations (up to 50 μM) ($p<0.001$). dichromate appeared to have the opposite effect on GR activity of which increased at the lowest (10-50 μM) and decreased at the highest concentration (100 μM). GR activity was significantly ($p<0.01$) induced relative to the control 132 and 352% by 10 and 50 μM Cr (VI), respectively (Table 2).

Effect of zinc: A substantial overall increase in activity GPx occurred in Zn treated cells at high concentration (100 μM) and was approximately 59% of the control activity. No significant change in GPx activity at low concentrations was detected. GST activity was increased 11% at low concentration up to 50 μM while an inhibitory effect of Zn was seen at concentrations of Zn equal to 100 μM. An overall decrease in activity of GR occurred in Zn treated cells at higher concentrations (100 μM) and decreased GR activity by 46%. A two fold increased in CAT activity at 10 μM Zn and was significantly differ from the control ($p<0.01$). Moreover, no further increase in CAT activity at high concentrations-up to 100 μM-was detected (Table 3)

Effect of Pb: The GPx activity significantly increased by about 37% at 100 μM Pb when compared to the control group ($p<0.01$). Simultaneously, the activity of GPx at low concentration was statistically not significant and decreased by about 30% when compared to the high

Table 3: *In vitro* zinc chloride effects on antioxidant enzymes activities in human red blood cells after 24 h exposure

	$ZnCl_2$ concentration (μM)#					
Enzyme	Control (0)	10	50	100	SE	Sig.
GPx (U/g Hb)	11.05[a]	10.91[a]	10.91[a]	17.55[b]	1.60	**
GR. (mU/g Hb)	1.24	1.13	1.04	0.67[b]	0.21	*
GSTs. (U/g Hb)	3.92[a]	4.45[b]	4.26[ab]	3.58[a]	0.11	***
CAT. (U/g Hb)	4.18	9.12[a]	9.68[a]	9.42[a]	1.45	**

Values are mean±SE, n = 5. *Significantly different from the control as p<0.05. **Significantly different from the control as p<0.01. *** Significantly different from the control as p<0.001. NS: Not Significant. #Human erythrocytes Antioxidant enzyme (GPx, GR, GSTs and CAT) activities were measured after 24 h of exposure to increasing concentrations of $ZnCl_2$

Table 4: *In vitro* lead chloride effects on antioxidants enzymes activities in human red blood cells after 24 h exposure

	$PbCl_2$ concentration (μM)#					
Enzyme	Control (0)	10	50	100	SE	Sig.
GPx (U/g Hb)	11.05[a]	11.82[a]	14.31[ab]	15.14[ab]	1.60	**
GR. (mU/g Hb)	1.24	1.94	1.78	1.78	0.47	NS
GSTs. (U/g Hb)	3.92[a]	4.30[b]	4.06[ab]	3.73[a]	0.10	*
CAT. (U/g Hb)	4.18	3.88	4.17	4.89	0.83	NS

Values are mean±SE, n = 5. *Significantly different from the control as p<0.05. **Significantly different from the control as p<0.01. NS: not significant. #Human erythrocytes Antioxidant enzyme (GPx, GR, GSTs and CAT) activities were measured after 24 h of exposure to increasing concentrations of $PbCl_2$

concentration. There were no significant changes in the GR activity of the red blood cells treated with Pb (p<0.01) when compared to the control. On the other hand, red blood cell GSTs activity showed a significant increase on lead exposure (p<0.01), while CAT remained statistically insignificant on lead exposure (Table 4).

Lipid peroxide production: The previous experiments showed that the maximal concentrations of metallic ions that caused no substantial cell disruption (little or no haemolysis) produced various modifications of the erythrocyte defense system against cellular oxidants. This defective antioxidant system could be responsible for membrane damage, including lipid peroxidation of PUFA. To further elucidate the mechanism of these alterations, human red blood cells were incubated in the presence of non-hemolytic concentrations of Cr, Cu, Pb and Zn then MDA was measured after 24 h later by TBA reactions. MDA erythrocyte concentration was significantly increased (p<0.001) at 50 and 100 μM Cu by about 37 and 211%, respectively, when compared to the control. There were no significant changes in MDA erythrocyte concentration between the control and low Cu concentration (10 μM). The effect of Cr (VI) on MDA was significantly high (p<0.05) at low concentration (10 μM) causing the later to increase by 159% and at 50 to 100 μM MDA was increased by 52%. Pb significantly increased the level of MDA by 53 and 138% at 10 and

Table 5: Malondialdehyde (MDA) concentration after 24 h of incubations of human erythrocytes in solutions of metal salts $CuCl_2$, $K_2Cr_2O_7$, $PbCl_2$ and $ZnCl_2$

	Metal concentration (μM)#					
Metal	Control (0)	10	50	100	SE	Sig.
Cu	0.978[a]	1.160[a]	1.336[b]	3.040[c]	0.095	***
Cr	0.978[a]	2.532[c]	1.421[b]	1.571[b]	0.260	*
Pb	0.978[a]	1.501[b]	2.329[c]	1.202[a]	0.076	***
Zn	0.978[a]	1.298[b]	1.501[c]	1.608[c]	0.082	**

Total MDA was measured in cells as TBA-reactive material. Values are expressed relative to cellular haemoglobin concentration. Values are mean±SE, n = 5. *Significantly different from the control as p<0.05. **Significantly different from the control as p<0.01. ***Significantly different from the control as p<0.001. NS: Not Significant. MDA was expressed as (μmole/L/g Hb)

50 μM, respectively (p<0.001), while no significant difference in MDA, when red cells exposed to high concentration. The MDA concentration significantly increased in red cells (p<0.01) as compared to the control by 33, 53 and 64% when treated with 10, 50 and 100 μM Zn, respectively (Table 5).

At concentrations ranged from 10-100 μM, the metal ions Cr, Cu, Pb and Zn caused the following increase in MDA level. Double in the case of cells treated with Cr or Zn (p<0.05) and more than triple with Cu and Pb (p<0.05). These results suggest that $CuC1_2$ and $PbC1_2$ are more toxic *in vitro* than $ZnCl_2$ or $K_2Cr_2O_7$, on the basis of its ability to generate membrane peroxides. This is in accord with the demonstrated ability of this metal to inhibit the major enzyme activities involved in detoxification of free radicals.

DISCUSSION

The response of human erythrocytes antioxidant enzymes to metallic salts exposure Cu, Zn, Pb and Cr was studied to explicate whether these can affect the CAT, GPx, GR and GSTs activities involved in the detoxification of Reactive Oxygen Species (ROS), thus leading to an excess of erythrocytes to cell membrane damage.

The results obtained in this study showed that treatment of human erythrocytes with Cu generally induces GPx activity at 100 μM but not statistically significant (p<0.05) and CAT significantly increase with gradual increase of Cu concentration (p<0.001). However, previous investigation on the effect of Cu resulted in a general decrease of GPx activity, especially at high concentration (1 mM) (Roche and BoGe, 1993). Such inhibition has been observed before in Carp tissues treated with Cu (Winston and DiGiulio, 1991) and in sea bass erythrocytes (Roche and BoGe, 1993). This numerical change in GPx can result from using small concentrations of metallic salts contrast to previous work done at high Cu concentration which can result from the oxidation of

intracellular Glutathione (GSH), by oxidants such Cu as soon as it enters the cell. In addition to serving as a substrate for the GSH peroxidase, GSH is also known as an intracellular Cu chelator (Aust *et al*., 1985). Also such activities of GPx and CAT may be explained by their influence on hydrogen peroxide as substrate which is formed in the process of dismutation of superoxide anion radicals (Shaikh *et al*., 1999). This action is followed by increased reduction of oxidized glutathione by glutathione reductase to form GSH (Mates, 2000). The GST enzyme has an important role in detoxification of the lipid hydroperoxides thus contributing to the protection of the cell integrity (Grose *et al*., 1987; Ochi *et al*., 1988; Ognjanovic *et al*., 1995). It has been shown, using sea bass erythrocytes incubated for 24 h that $CuSO_4$ and $ZnCl_2$ cause an overall decrease in these antioxidant enzymes activities (Winston and Di Giulio, 1991).

We have shown in the present study that zinc leads to increased MDA while causing a reduction in GPx activity at low concentration and induction at high concentration. Zinc is a component of Cu-Zn SOD and the increased presence of zinc in the red cell has caused a mild inhibition of this enzyme. The increasing dose of zinc showed an induction effect on catalase and GSTs activity, whereas GR activity was only reduced at the highest dose tested. Because zinc is a nonredox-active element, it does not respond to stress as easily as other redox-active elements like copper or iron (Klotz *et al*., 2003; Kuppusamy *et al*., 2005).

Since Cr is reduced as soon as it enters the cell by GSH. In addition to serving as a substrate for GSH peroxidase, GSH is also known to function as an intracellular copper chelator (Aust *et al*., 1985). Among other possibilities, the substitution of covalently cysteine-bound selenium by another metallic cation can be advanced as a cause of the possible inhibition of MnSOD, or of excessive production of free radicals (Winston and Di Giulio, 1991). In yeasts, the major effect of metal exposure is overall stimulation of GPx activity (Galiazzo *et al*., 1988); such enhanced activity was found in isolated bass erythrocytes at the lowest concentrations of Cr (VI).

Many evidence suggest that cellular damage mediated by oxidative stress may be involved in some of the pathologies associated with lead toxicity (Adonaylo *et al*., 1999; Sandhir *et al*., 1995). Lead is one of the most widely used metals in human history, industry and release into the environment as an exhaust emission product (Shotyk *et al*., 1998). This has made lead a serious risk factor to human through lead intoxication and poisoning (Juberg *et al*., 1997). Exposure to low levels of lead has been associated with cellular damage mediated by Reactive Oxygen Species (ROS), which may be involved in the pathology associated with lead intoxication (Bechara *et al*., 1993; Hermes-Lima *et al*., 1991). Lead stimulated oxidative hemolysis of erythrocytes, decreased erythrocyte SOD activity and accelerated conversion of oxyhemoglobin to methemoglobin (Gurer and Ercal, 2000). The malondialdehyde levels in blood were strongly correlated with lead concentration in exposed blood (Jiun and Hsien, 1994). This study showed that in human erythrocytes exposed to lead, the antioxidant enzymes activities of the GPx, GR, GST were remarkably higher than that in nonexposed human erythrocytes. Such activities were observed in erythrocytes from the workers exposed occupationally to lead and were remarkably higher than that in nonexposed workers (Monteiro *et al*., 1985).

The detoxication effect mediated by the antioxidant system including the glutathione and glutathione-detoxication enzyme such as CAT,GR, GSTs and GPx might be attenuated by heavy metals as a part of either intra or extracelluar oxidative stress induced mechanism. The increase of lipid peroxidation levels indicated that the metal ions caused oxidative damage to cell membranes. Thus peroxidation of cell membrane phospholipids and accumulation of lipid peroxides are expected to change the membrane fluidity and consequently the membrane function. The mechanism and the toxic effects of ions on human erythrocytes may be variable. Cu and Pb-induced membrane lipid oxidation may be different from that of Zn and Cr ions in the human erythrocytes

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