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Full Length Research Paper

Potassium antimony tartrate affects the chemical and metabolic status of glutathione in human blood: *Ex vivo* studies

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Toxicity of metal elements and their salts are compromised usually because of therapeutic needs. Glutathione (GSH), a master antioxidant of human body, constitutes a major portion of natural redox system. GSH also strengthens the immune system and paces healing after surgery or infection. Compounds of antimony (Antimonials) are useful in the treatment of leishmaniasis and schistosomiasis. As the compounds cause cardiotoxicity and pancreatitis, so regular monitoring for toxicity is required when they are used therapeutically. Therefore, it is of great interest to evaluate the *ex vivo* effect of potassium antimony tartrate (PAT) on the status of GSH in the blood components (plasma and cytosolic fraction) of healthy human volunteers. Interaction of the GSH with PAT was carried out *ex-vivo* as a surrogate to *in vivo* effects. The concentration of GSH was measured with ultraviolet (UV)-visible spectrophotometer, using a previously published method (Ellman et al., 1961). The level of GSH was lowered in plasma and cytosolic fractions upon addition of PAT to whole venous blood; the lowering of GSH content was dependent upon PAT concentration and time of incubation. The reduction in GSH level in blood plasma and intracellular components may be either due to formation of PAT-SG complex or conversion of reduced GSH to its oxidized form, glutathione disulphide (GSSG).

Key words: Glutathione (GSH), potassium antimony tartrate, Ellman's method, oxidative stress.

INTRODUCTION

Glutathione (gamma-glutamyl-cysteinyl-glycine, GSH) is an endogenous tripeptide thiol, which contributes appreciably to antioxidant defense system, nutrient metabolism and regulation of cellular events including gene expression, cell proliferation and apoptosis, signal transduction and immune response (Sen, 1998). In healthy cells and tissues, the GSH is exclusively found in its reduced form. The ratio of reduced GSH to oxidized glutathione, which is glutathione disulphide (GSSG) within the cells, is often used scientifically as a measure of cellular toxicity (Pastore et al., 2003). GSH deficiency adds to oxidative stress, which plays a key role in aging and pathogenesis of many diseases. The GSH has a vital role in maintenance of body defense system, fighting against diseases and helping in preventing metal and drug poisoning. A large number of investigators (Bashandy et al., 2011; Fahmy and Hamdi, 2011; Etuk et al., 2009) have proved the vital protective role of GSH in poisoning caused by plant extracts. Thus, new knowledge of the nutritional regulation of GSH metabolism is critical for the development of effective strategies to improve human health and to treat the above mentioned disease conditions (Wu et al., 2004).

In addition, the bio-coordination chemistry of antimony has been extensively explored in the backdrop of historical use of this metal in medicine. The clinically used and biologically active form of antimony is Sb^{3+}, while Sb^{5+} is

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used as a pro-drug, which is converted to its active form Sb^{3+} inside the cells (Goodwin and Page, 1943). Antimony and its compounds have many therapeutic applications, including antischistosomal, antiprotozoal and expectorant in the form of potassium antimony tartrate (PAT) and in veterinary practice as a skin conditioner. The potassium antimony (III) tartrate used in the present study has the capability of bimolecular binding to GSH, trypanothione and nucleotides with the formation of binary and ternary complexes. This trend of PAT binding allows it to be trafficketed in cells (Ge and Sun, 2007). Previously conducted studies revealed that Sb^{5+} inhibits energy metabolism and macromolecular biosynthesis via inhibition of glycolysis and fatty acid β-oxidation (Berman et al., 1987; Sereno et al., 2001). SB^{3+} also inhibits trypanothione reductase *in vitro*, but the significance of this observation still remains unclear (Cunningham and Fairlamb, 1995). Some studies (Murray et al., 1988) have reported that Sb^{3+} treated amastigotes undergo death by an apoptotic-like mechanism involving DNA fragmentation and externalization of phosphatidylserine by a caspase-independent mechanism.

In spite of its therapeutic value, it has also toxicological profile. The impact of antimony on human health from toxicological point of view is not exactly known, but it is now clear that its trivalent form is responsible for mediation of their toxic effects (Roberts et al., 1995; Shaked-Mishan et al., 2001). The metalloid has carcinogenic potential and also acts as clastogenic (Groth et al., 1986; Gebell, 1997). Treatment with PAT has been reported to cause life-threatening arrhythmias (Yuri et al., 2006). Antimony (III) oxide (by inhalation) has been found to produce lungs cancer in rats. Increased GSH levels and induction of anti-stress proteins were found associated with protection against oxidative stress that is induced by antimony (Tirmenstein et al., 1995). Thus, it is of interest to evaluate the interaction of this metal based drug with glutathione in *ex vivo* as a model of in *vivo* reaction to establish further scientific data which can strengthen the knowledge about the toxicological profile of antimony and the role of GSH in the improvement of the current antimony therapy along with control on growing problem of its resistance.

MATERIALS AND METHODS

Chemicals, reagents and equipments

GSH (Fluka), 5,5-Dithiobis, 2 nitrobenzoic acid (DTNB) (Sigma), sodium hydroxide (Fluka AG), sodium chloride (Merck), disodium edetate (Riedel Dehean AG Sleeze Hannover), potassium dihydrogen phosphate (Merck), HCl 35% (Kolchlight), PAT (Across, Belgium), distilled water (double refined), chloroform (Merck), ethanol (Merck), ultraviolet (UV)-Visible spectrophotometer: Model 1601 (Schimadzu, Japan), pH meter: Model NOV-210 (Nova Scientific Company Ltd. Korea), analytical balance AX 200 (Schimadzu, Japan), centrifuge H-200 (Kokusan Ensink Company Japan), Eppendorf tubes (Plastic, 10l) oven: Memmert Model U-30,854 (Schwa Bach, Germany) and magnetic stirrer were used in the study.

Methods

Preparation of solutions

0.9% W/V sodium chloride solution was prepared by dissolving exactly 900 mg of NaCl in 100 ml of water. 50 ml solution of PAT (1 mM) was prepared by dissolving 18.96 mg of PAT in distilled water. GSH standard solution (1 mM)) was prepared by dissolving 30.74 mg of GSH in 100 ml of 0.1 N HCl. DTNB (1mM) was prepared by dissolving 39.6 mg of DTNB in 100 ml of phosphate buffer. Phosphate buffer (0.2 M) with pH 7.6 was prepared by mixing 42.2 ml of NaOH (0.2 M) and 50 ml mono-basic potassium phosphate solution (0.2 M) and making the volume upto 200 ml with distilled water. pH was measured and adjusted using pH-meter (Accumet meter, Denver instrument company, USA).

Preparation and isolation of blood components

Isolation of plasma

Fresh venous blood (12 ml) treated with 0.5 mM Na- ethylene diamine tetraacetic acid (EDTA) 500 µl to prevent clotting was collected from healthy human volunteers. Each time 1.0 ml venous blood was taken and mixed with 1.0 ml of different concentration of PAT (6.67 to 33.34 µM) solution and incubated for 10 min. Each of the 2 ml sample containing blood and PAT solution in 1:1 ratio was then centrifuged at 10000 rpm for 5 min. The supernatant fluid 0.8 ml (plasma) was removed with Pasteur pipette, transferred to sample tubes and kept on ice ball till use and the packed cells were immediately further processed for cytosolic fraction. A control containing 1 ml of venous blood and 1.0 ml of 0.9% w/v NaCl solution was incubated as mentioned above and also centrifuged for isolation of plasma and cytosolic fraction.

Isolation of cytosolic-fraction of blood

The packed cells were washed twice with isotonic saline (0.9% w/v NaCl) solution and the blood cells were lysed at 4°C with an equal volume (1:1) of distilled water for 1 h. After 1 h lysis at 4°C, 0.8 ml of cold mixture of chloroform- ethanol (3:5 V/V) at 0°C was added to 2 ml of lysed cells to precipitate the hemoglobin, followed by 0.3 ml of distilled water. The resulting mixture was centrifuged as before and the pale yellow clear supernatant (cytosolic fraction) was removed by Pasteur pipette and transferred to sample tubes and stored on ice till use.

Determination of glutathione

(1) Plasma glutathione (Extracellular)
(2) Lysate Glutathione (Intracellular)

All GSH estimations were carried out with a previously published method (Ellman et al., 1961). Each time 2.3 ml phosphate buffer was added to 0.2 ml of the sample (plasma or cytosolic fraction of blood) followed by the addition of 0.5 ml of DTNB (1 mM). This mixture was transferred to the spectrophotometer cell. The reference cell contained 0.2 ml of the sample and 2.8 ml of the phosphate buffer. DTNB blank consisting of 2.5 ml buffer, 0.5 ml DTNB was measured against a reference cell containing 3 ml buffer. All measurements were taken after 5 min at 412 nm. The GSH contents were calculated using the standard curve.

Absorbance readings were taken using UV-visible

Table 1. Effect of different concentrations of PAT on the chemical status of GSH at different time intervals in plasma.

Absorbance of 5,5-dithiobis,2-nitrobenzoic acid (DTNB) blank solution was 0.069 at 412 nm.													
S/N	Conc. of PAT (µM)	Real abs*/ conc. at 0 h		Real abs*/ conc. at 1 h		Real abs*/ conc. at 2 h		Real abs*/ conc. at 3 h		Real abs*/ conc. at 4 h		Real abs*/ conc. at 5 h	
		Abs.	Conc.	Abs.	Conc.	Abs.	Conc.	Abs.	Conc.	Abs.	Conc.	Abs.	Conc.
1	6.67	0.381	2.36	0.365	2.27	0.339	2.12	0.313	1.96	0.298	1.88	0.263	1.67
2	13.34	0.353	2.19	0.335	2.09	0.317	1.99	0.301	1.89	0.276	1.75	0.249	1.59
3	20.00	0.321	2.01	0.307	1.93	0.287	1.81	0.272	1.73	0.251	1.60	0.232	1.49
4	26.67	0.299	1.88	0.279	1.77	0.254	1.62	0.239	1.53	0.223	1.44	0.213	1.38
5	33.3	0.269	1.71	0.248	1.59	0.223	1.44	0.217	1.41	0.201	1.31	0.192	1.26
Real abs./conc. for blank GSH		0.644	3.89	0.644	3.89	0.644	3.89	0.644	3.89	0.644	3.89	0.644	3.89

*Real absorbance = Absorbance of mixture - Absorbance of DTNB blank solution. Conc.: Concentration. abs.:Absorbance.

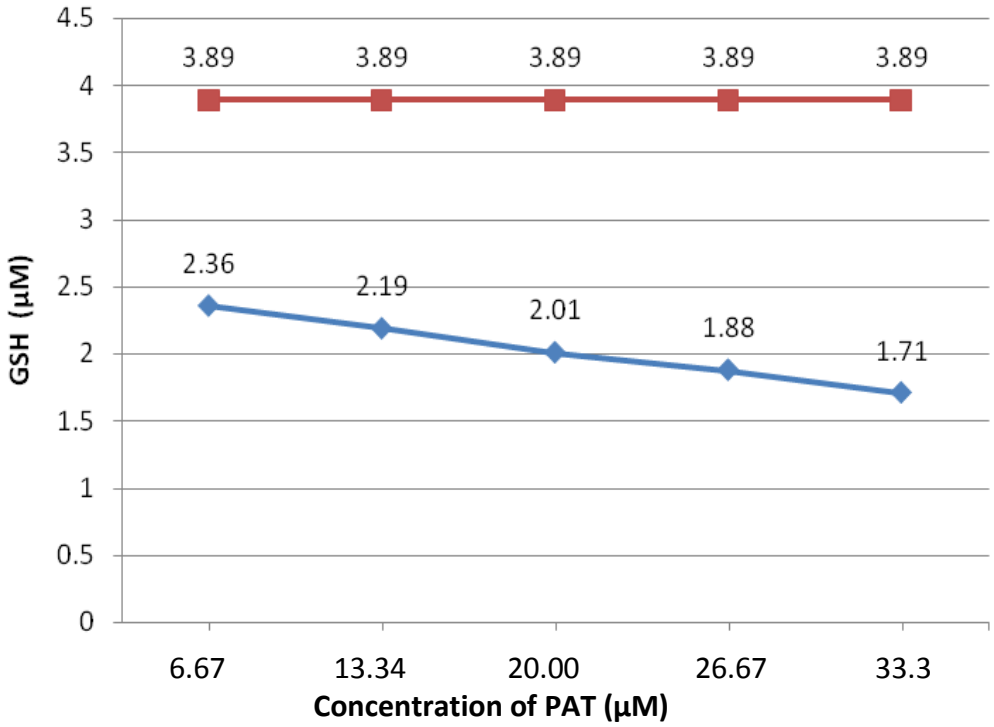

Figure 1. Effect of different concentrations of PAT on GSH content in extracellular plasma. ■, Control (1 ml 0.9% w/v NaCl/1 ml of blood) represented by square symbol; ♦, PAT (6.67 to 33.33 µM) represented by diamond symbol. Results are the mean ± SEM of three experiments.

spectrophotometer (Model 1601, Shimadzu).

Standard curve

Standard curves were built for determination of GSH levels in samples. Quality control solutions of different concentrations of GSH ranging from 13.33 to 66.66 µM were processed with an already published method (Ellman et al., 1961).

RESULTS

PAT effect on GSH content of plasma fraction of blood

The GSH content of plasma fraction of blood decreased proportionally to the concentration of PAT and its time of exposure as shown in Table 1 and Figure 1.

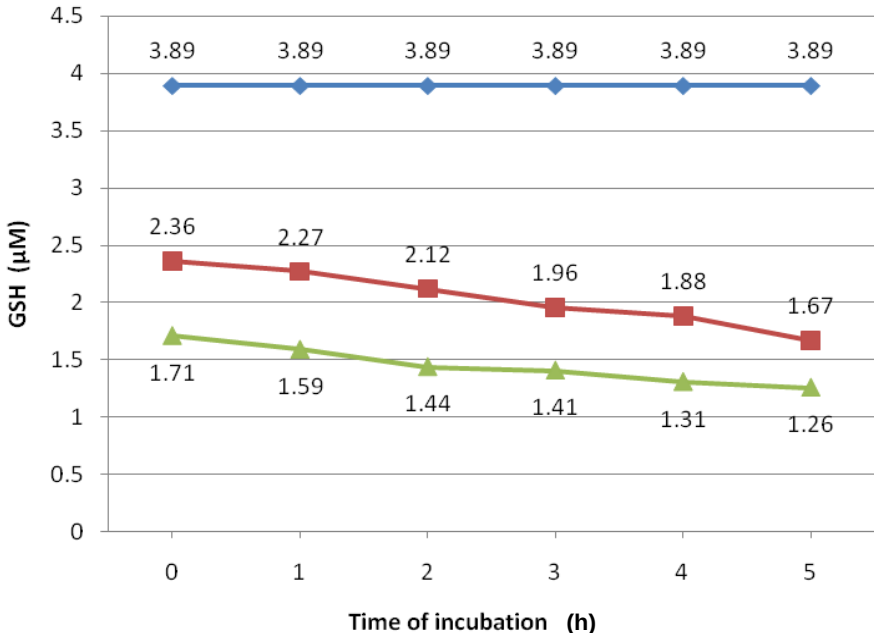

Figure 2. Effect of PAT concentration on the extracellular plasma GSH content with time incubation period (0 to 5 h). ♦, Control (1 ml 0.9% w/v NaCl/1 ml of blood) represented by diamond symbol; ■, PAT (6.67 µM) represented by square symbol; ▲, PAT (33.33 µM) represented by triangle symbol. Results are the mean ±SEM of three experiments of plasma GSH.

Table 2. Effect of different concentrations of PAT added to blood before separation on the chemical status of GSH with time in cytosolic fraction.

Absorbance of 5,5-dithiobis,2-nitrobenzoic acid (DTNB) blank solution was 0.065 at 412 nm.													
S/N	Conc. of PAT (µM)	Real abs*/ conc. at 0 h		Real abs*/ conc. at 1 h		Real abs*/ conc. at 2 h		Real abs*/ conc. at 3 h		Real abs*/ conc. At 4 h		Real abs*/ conc. at 5 h	
		Abs.	Conc.	Abs.	Conc.	Abs.	Conc.	Abs.	Conc.	Abs.	Conc.	Abs.	Conc.
1	6.67	0.481	2.94	0.451	2.77	0.429	2.64	0.403	2.49	0.378	2.34	0.354	2.20
2	13.34	0.453	2.78	0.424	2.61	0.407	2.51	0.385	2.38	0.356	2.22	0.332	2.08
3	20.00	0.436	2.68	0.401	2.48	0.376	2.33	0.353	2.19	0.339	2.12	0.312	1.96
4	26.67	0.413	2.55	0.379	2.35	0.351	2.19	0.327	2.05	0.313	1.97	0.291	1.84
5	33.3	0.389	2.41	0.346	2.16	0.329	2.06	0.305	1.92	0.283	1.79	0.263	1.67
Real abs./conc. for Blank GSH		0.560	3.41	0.560	3.41	0.560	3.41	0.560	3.41	0.560	3.41	0.560	3.41

*Real absorbance = Absorbance of mixture - Absorbance of DTNB blank solution. Conc.: concentration; abs.:Absorbance.

A statistically significant change ($p < 0.05$) in the content of plasma GSH from control was observed. Plasma GSH content at time intervals (0 to 5 h) was also measured when different concentrations of PAT (6.67 to 33.33 µM) were added. The GSH content of extracellular plasma decreased at different time intervals (Figure 2). These results showed that the decreases in plasma GSH content were both PAT concentration and exposure time dependent.

PAT effect on GSH content of intracellular cytosolic fraction of blood

Each time 1.0 ml of PAT (6.67 to 33.33 µM) solution was added to 1.0 ml venous blood. Upon addition of PAT to venous blood, measurement of intracellular cytosolic GSH content showed gradual decrease in GSH content as shown in Table 2 and Figure 3. Intracellular Cytosolic GSH content was also measured at 0 to 5 h after the

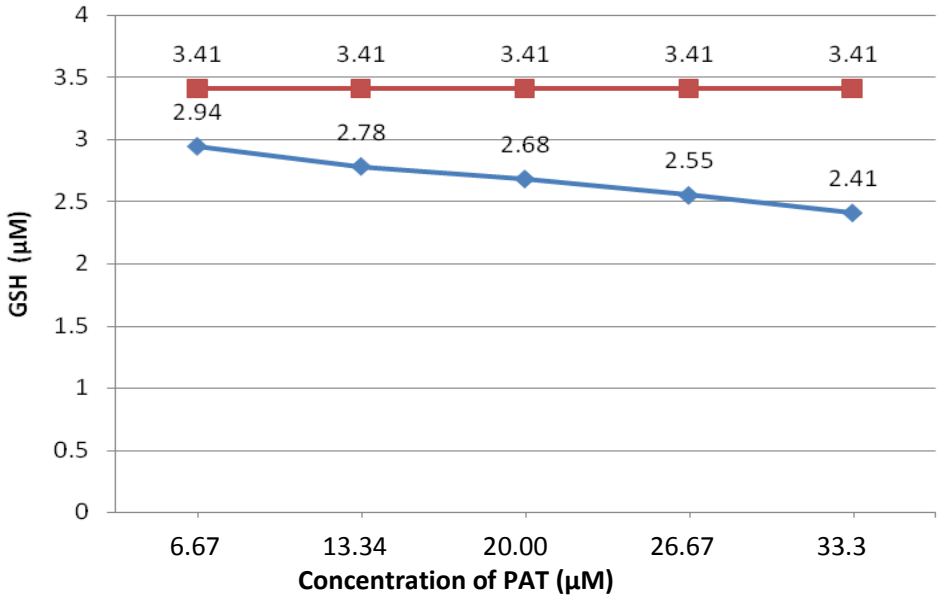

Figure 3. Effect of PAT on GSH content of cytosolic fraction. ■, Control (1 ml 0.9% w/v NaCl/1 ml of blood) represented by square symbol; ♦, PAT (6.67 to 33.33 µM) represented by diamond symbol. Results are the mean ±SEM of three experiments of cytosolic fraction GSH.

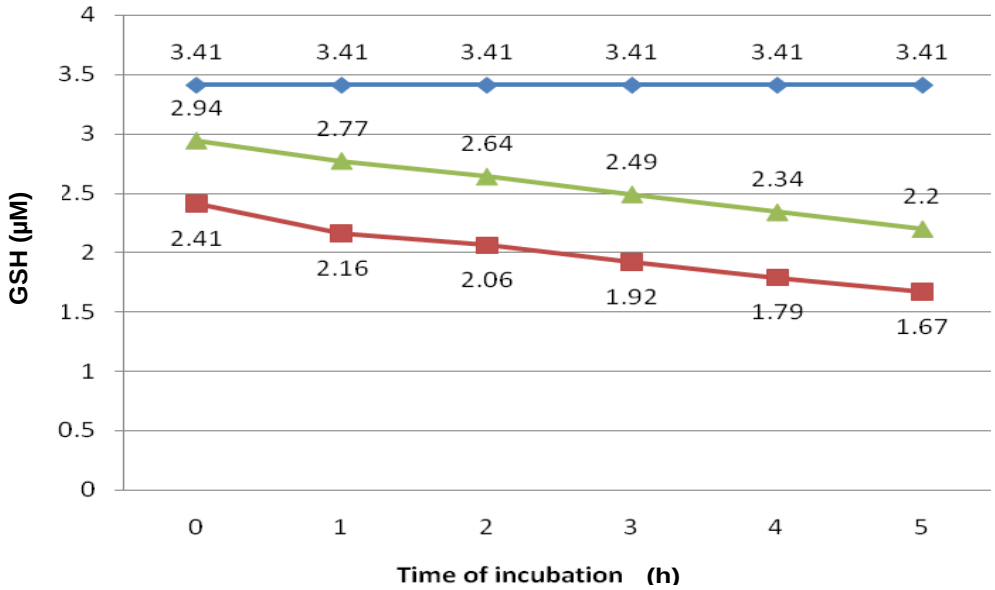

Figure 4. Effect of PAT on intracellular cytosolic fraction GSH content with exposure time (0 to 5 h). ♦, Control (1 ml 0.9% NaCl/1 ml of blood) represented by diamond symbol; ■, PAT (6.67 µM) represented by square symbol; ▲, PAT (33.33 µM) represented by triangle symbol. Results are the mean ±SEM of three experiments of cytosolic fraction GSH.

addition of PAT to venous blood. A statistically significant change ($p < 0.05$) in the content of cytosolic fraction GSH from control was observed. Results show the time dependent decrease in GSH content as shown in Table 2 and Figure 4.

DISCUSSION

GSH plays an important role in the biological processing of antimonial drugs (Sun et al., 2000). Therefore, we studied the effect of the antileishmanial drug PAT on

GSH level in plasma and cytosolic fraction of human venous blood. The concentrations of PAT used in the present study ranged from 6.67 to 33.34 µM, which contain 1624.1×10^{-6} to 8118.3×10^{-6} mg/ml of antimony while the therapeutic concentration of antimony that is required for the proper treatment of leishmania patient ranges from 1 to 12 mg/ml (Chulay et.al., 1988). The effect of antimony on the chemical and metabolic status of GSH was studied in terms of determination of concentration of GSH at λ_{max} 412nm. This λ_{max} (412 nm) is being used for the determination of GSH concentration in samples according to a published method (Ellman et al., 1961).

Previously, it has been found that addition of GSH to cultured cardiac muscle cells provides protection against the lethality of PAT (Tirmenstein et al., 1995). The exogenous GSH provides protection due to the binding of antimony by sulfhydryl (SH) groups present in GSH (Tirmenstein et al., 1997). This shows that increased level of GSH in vivo produced by low-level PAT exposure is a factor in the protection against subsequent lethal concentrations of PAT. Thus, increased level of GSH and the induction of stress protein have been found to be associated with protection against oxidative stress that is induced by antimony (Trimenstein et al., 1995). Accordingly, the interaction of this metal based drug with glutathione *in vitro* as a model for *in vivo* reaction may establish further scientific data and strengthen our knowledge about the toxicological profile of antimony and the role of GSH in the protection of our body from its harmful effects.

According to our findings, the antimony in the form of potassium antimony tartrate induced the depletion of GSH in a dose dependent manner. The results also show positive correlation between the exposure time of GSH to the above given concentrations of PAT and the depletion of GSH as the time passed from 0 to 5 h. Furthermore, the organo-metallic compounds or salts like PAT are hydrophilic and hence more effective on extracellular side (plasma) as compared to intracellular side (Cytosolic fraction), so the oxidation and or conjugation of GSH have been found to be more pronounced on plasma fraction as compared to cytosolic fraction (Haldar et al., 2011).

The exact mechanism of the reaction between PAT and reduced GSH still needs to be fully investigated, but the formation of $Sb(GS)_3$ complex may be proposed. This interpretation is also supported by a recent study that established the formation of $Sb(GS)_3$ from Sb^{3+} and GSH (Sun et al., 2000), in which the deprotonated SH group of the cysteine residue is found to be the exact binding site for Sb^{3+}. The rate of exchange of GSH between its free and Sb-bound form is pH-dependent, and, therefore, the study was carried out at pH 7.6. Such facile exchange may be important in the transport of Sb(III) in various biofluids and tissues *in vivo* (Sun et al., 2000).

The main soluble forms of pentavalent and trivalent antimony in the pH range of 2 to 8 are assumed to be SbO_3 and $HSbO_2$, respectively and the half-reaction for antimony reduction occurs as follows (Pitman et al., 1957).

$$SbO_3^- + 3H^+ + 2e^- \longrightarrow HSbO_2 + H_2O \quad (1)$$

Based on these assumptions, the following oxidation-reduction reaction can be proposed:

$$SbO_3^- + H^+ + 2GSH \longrightarrow HSbO_2 + GS\text{-}SG + H_2O \quad (2)$$

Here, GSSG is the oxidized form of GSH.

The formation of the $Sb(GS)_3$ complex may be described by the following reaction:

$$HSbO_2 + 3GSH \longrightarrow Sb(GS)_3 + 2H_2O \quad (3)$$

Therefore, from Reactions 2 and 3, the following general reaction can be proposed:

$$SbO^{3-} + 5GSH + H^+ \longrightarrow Sb(GS)_3 + GS\text{-}SG + 3H_2O \quad (4)$$

Previous studies have shown that there is no binding of antimony in the form of PAT to the oxidized form of GSH, which is GSSG under similar conditions which indicates that antimony binding to GSH occurs at the free thiolate sulfur (Sun et al., 2000). Antimony has also little effect on the overall conformation of GSH, what allows the complex to be easily recognized by cell receptors for GSH and provides a transport mechanism for Sb^{3+} (Gebel, 1997; Gyurasics et al., 1992). As excretion of antimony has been reported to be accompanied by co-transport of three molecules of GSH (Gyurasics et.al., 1992), which is again consistent with the formation of $Sb(GS)_3$ complex as shown in our studies. The reported resistance of parasites to antimony is probably due to complexation of the metal with GSH and pumping out of the complex $Sb(GS)_3$ from the cells or parasites by an ATP-coupled pump (Dey et al., 1996). The above data also gives clear picture of antimony excretion which is conjugated with GSH and then excreted in the bile (Gebel, 1997; Gyurasics et al., 1992; Winship, 1987).

Conclusion

The PAT caused depletion of GSH in blood plasma and intracellular components which may ultimately lead to defective redox system and consequently serious side effects. The lowering of reduced GSH content may be either due to formation of PAT-SG complex or oxidation of GSH to GSSG.

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