Full Length Research Paper

Human blood glutathione (GSH) as a tool for arsenic detoxification

Naseem Ullah*, Muhammad Farid Khan and Muhammad Mukhtiar

Department of Pharmaceutical Chemistry, Gomal University, D. I. Khan, KPK Pakistan.

Accepted 12 September, 2011

Arsonic is a major threat to a large part of the population due to its carcinogenic nature. The toxicity of arsenic emerges from glutathione (GSH) depletion caused by arsenic with unknown mechanism. GSH depletion leads to apoptosis, lipid peroxidation and eventual cell death. The present study was designed to provide insight into the extent of changes in GSH level by arsenic. Plasma and cytosolic fraction were investigated for determination of changes in GSH metabolic status caused by arsenic in the form of arsenic trioxide (ATO). The depletion of GSH level was found to be positively correlated with increasing parameters, that is, arsenic concentration and time of incubation. Our findings show that changes in GSH status produced by arsenic could be due to adduct (As-(SG)₃) formation. This change in GSH metabolic status provides information regarding mechanism of toxicity of ATO. These findings are important for the rational design of antidote for the prevention of arsenic induced toxicity.

Key words: Arsenic trioxide (ATO), glutathione (GSH), dithiobisnitrobenzoic acid (DTNB), plasma, cytosolic fraction (C.F).

INTRODUCTION

Arsonic (As) is a widespread environmental toxin. It enters the organisms by dermal contact, inhalation, or ingestion of contaminated drinking water and affects nearly entire organ systems of the body (Ratnaikc, 2003). Arsonic compounds are quite effectively incorporated from the gastrointestinal tract. Arsenate (V) is absorbed to roughly 60%, trivalent arsenic to 80% and natural organoarsenicals to nearly 100% (Zielhuis and Wibowo, 1984).

Glutathione (GSH) is the most prevalent low molecular weight cellular thiol in mammalian cells. In addition to having a major cellular role in oxidative status, there is an important function of GSH in sequestering toxic, electrophilic xenobiotics (Boyland and Chasseaud, 1969; Chasseaud, 1976). Nucleophilic reactivity of GSH is based on conjugation of its thiol group with electrophilic compounds (Commandeur et al., 1995). This characteristic underlies its potent antioxidant action and

enzyme cofactor properties, and supports a complex thiol-exchange system, which hierarchically regulates cell activity. GSH levels in human tissues normally range from 0.1 to 10 millimolar (mM), most concentrated in the liver (up to 10 mM) and in the spleen, kidney, lens, erythrocytes, and leukocytes (Bremer et al., 1981). Oxidative stressors that can deplete GSH include ultraviolet and other radiation (Cai et al., 2000), viral infections (Kidd, 1997; Look et al., 1997), environmental toxins, household chemicals, and heavy metals (Kidd, 1997) surgery, inflammation, burns, septic shock (Luo et al., 1998; Spies et al., 1994) and dietary deficiencies of GSH precursors and enzyme cofactors (Whitcomb and Block, 1994).

Most of the toxicological features of arsenic described here may be explained by a high affinity to sulfhydryls resulting in an inhibition of a great number of enzymes. Investigations at the cellular and molecular levels reveal that As enhances production of reactive oxygen species (like, superoxide and hydrogen peroxide), causes lipid peroxidation, enhances oxidation of proteins, enzymes as well as DNA (Yamauchi et al., 2004; Kitchin, 2001), disrupts mitosis and promotes apoptosis (States et al.,

*Corresponding author. E-mail: naseemscholar@gmail.com.
Tel: 03239989477.

2002). These data suggest that ATO-induced apoptosis is attributed primarily through the depletion of GSH.

Glutathione and enzymes related to GSH synthesis comprise a system that maintains the intracellular reducing environment and acts as a primary defense against excessive generation of harmful ROS. One of the important mechanisms of arsenic induced disorders is its ability to bind with sulfhydryl group (-SH) containing molecules. Trivalent inorganic arsenicals, such as arsenite, readily react with reduced glutathione (GSH) and cysteine and decrease their bio-availability (Thomas et al., 2001). Our study will further provide the detailed information regarding the extent of changes occurring in GSH level in plasma and cytosolic fraction, when subjected to different ATO concentrations and different time fixations.

MATERIALS AND METHODS

The materials includes L. glutathione (GSH) (Fluka), DTNB (Sigma), sodium hydroxide (Fluka AG), sodium chloride (Merck), disodium edetate (Riedel Dehean AG Sneeze Hannover), potassium dihydrogen phosphate (Merck), HCl 35% (Kolchlight), arsenic trioxide (Across, Belgium), distilled water (Double Refined), chloroform (Merck), ethanol (Merck), spectrophotometer: UV. visible, 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's tubes (Plastic, 101) oven: Memmert model U-30,854 (Schwa Bach, Germany) and magnetic stirrer. Were purchased from local market.

Preparation of stock solution

To make 0.9%-NaCl solution, 90 mg of pharmaceutical grade NaCl was added to D/W quantity sufficient (q.s.) 100 ml. Arsenic trioxide (ATO), 2 mM solution contained 19.8 mg of ATO in D/W q.s. 50 ml, which was again gradually diluted to get 10 different (0.2, 0.4, 0.6, 0.8, 1.00, 1.2, 1.4, 1.6, 1.8 and 2.00 mM) concentrations of ATO. GSH (1 mM) standard solution was obtained through dissolving 15.375 mg of GSH in 50 ml of phosphate buffer (pH 7.6). 19.8 mg of 5-5, dithiobis 2-nitrobenzoic acid (DTNB) was added to phosphate buffer (pH 7.6) to get 50 ml of 1 mM solution of DTNB.

Isolation of plasma and cytosolic fraction

Plasma

Fresh venous blood (12 ml) was collected from a healthy human volunteer in a heparinized test tube. ESR of this blood sample was 12 mm, clotting time 4 min and Hb was 15.4 g/dl. Hematocrit included blood cells 45%, plasma 55% and RBCs count was 5.2 million/mm³. From this blood sample, each time, 1 ml was taken and added to 1 ml of each of the 10 concentrations of the ATO solution resulting 10 mixtures having different concentrations of ATO. All the mixtures were shaken and incubated for 5 min. Each of these 2 ml samples containing blood and ATO in 1:1 ratio were centrifuged at 10,000 rpm for 5 min. The respective supernatant (Plasma) was carefully pipetted upto 0.8 ml from each mixture and transferred to separate sample tubes. The remaining packed cells fractions were processed further for the collection of lysate or

cytosolic fraction.

Cytosolic fraction

The packed cells fractions were washed twice with 0.9% NaCl (isotonic) solution and then added drop wise D/W equal in volume to lyse RBCs. Then 0.6 ml of cold mixture of chloroform and ethanol (3:5 v/v) was incorporated at 0°C to precipitate hemoglobin. The resultant fractions were centrifuged and pale yellow supernatant (Cytosolic fraction) was collected from each mixture and transferred to separate sample tubes.

Plasma and cytosolic fraction controls

Plasma control was prepared through mixing blood and 0.9% NaCl solution in 1:1 ratio and then centrifuged to collect plasma portion as before.

Control for cytosolic fraction was prepared through mixing blood and 0.9% NaCl solution in 1:1 ratio and then centrifuged to collect cytosolic fraction portion as before.

Experimental design

Through centrifugation, plasma and cytosolic fraction were collected in separate sample tubes. The concentration dependent effect of ATO (0.2, 0.4, 0.6, 0.8, 1.00, 1.2, 1.4, 1.6, 1.8 and 2.00 mM) on GSH level was studied in plasma and cytosolic fraction each. The final ATO concentration (in each 10 samples of plasma and 10 samples of cytosolic fraction) was 100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 µM. Data for effect of ATO on GSH level at different time fixations (00, 30, 60 and 90 min) was also collected. The time course studies were carried for each concentration of ATO, from 100 to 1000 µM.

Experimental parameters

Assay of plasma and cytosolic fraction incubated with different ATO concentrations and at different time fixations was made for estimation of effective toxicity marker compound, glutathione, in reduced form (GSH).

Determination of GSH concentration

The reduced glutathione level was determined by the method of Ellman (1959).

0.2 ml from each of the samples of the plasma/cytosolic fraction (having different ATO concentration) was added to 2.3 ml of phosphate buffer (pH 7.6). Then 0.5 ml of reagent (DTNB) was added. The absorbance was determined at 412 nm against respective control, where ATO had not been added. The concentration of GSH was expressed as µM.

RESULTS

Assay of plasma-GSH level

Change in plasma-GSH level at different ATO concentrations: GSH level was determined in different plasma samples, each one having varied ATO concentrations (100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000

Table 1. Effect of Different concentrations of arsenic trioxide (0.2 to 2mM) on the chemical status of glutathione (GSH) with time in plasma (arsenic trioxide was injected into whole blood before its separation and incubated for 30 min).

S/N	Concentration of arsenic trioxide (μM)	Average ABS at 0 min	Concentration (μM) of GSH at 0 min	Average ABS at 30 min	Concentration (μM) of GSH, at 30 min	Average ABS at 60 min	Concentration (μM) of GSH, at 60 min	Average ABS at 90 min	Concentration (μM) of GSH, at 90 min
1	100	0.442	38.1	0.431	37.2	0.413	35.7	0.394	34.2
2	200	0.393	34.1	0.378	32.9	0.362	31.6	0.349	30.5
3	300	0.362	31.6	0.344	30.1	0.327	28.7	0.312	27.5
4	400	0.328	28.8	0.315	27.7	0.303	26.7	0.287	25.4
5	500	0.303	26.7	0.288	25.5	0.263	23.5	0.251	22.5
6	600	0.284	25.2	0.267	23.8	0.249	22.3	0.234	21.1
7	700	0.261	23.3	0.243	21.8	0.227	20.5	0.219	19.8
8	800	0.239	21.5	0.216	19.6	0.189	17.4	0.172	16.0
9	900	0.217	19.7	0.194	17.8	0.178	16.5	0.157	14.8
10	1000	0.196	18.0	0.175	16.2	0.161	15.1	0.143	13.6
Plasma control		0.647	54.9	0.647	54	0.647	54.9	0.647	54.9

μM). The spectrophotometric analysis showed marginal drop in GSH level (38.1 μM) with the initial ATO concentration, that is, 100 μM compared to the control plasma-GSH (54.9 μM). The GSH level seemed continuously declining in other plasma samples which contained increasing ATO concentrations. The maximum used ATO concentration (1000 μM) brought drop in GSH level up to 18.00 μM compared to the control plasma-GSH (54.9 μM) as shown in Table 1 and Figure 1.

Change in plasma-GSH level by ATO at different time fixations: Each plasma sample having one of the different ATO concentrations (100 to 1000 μM) was incubated upto different fixations time (0-, 30-, 60- and 90-min). By extending incubation period, the plasma-GSH level was further decreasing, the maximum decrease in plasma-GSH level being with the maximum provided time as shown in Table 1 and Figure 2.

Assay of C.F-GSH level

Change in C.F-GSH level at different ATO concentrations: Different C.F samples were also assayed for GSH determination containing different ATO concentrations (100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 μM). C.F-GSH level showed prominent drop (37.6 μM) with the initial of the used concentration (100 μM) compared to the control C.F-GSH (67.5 μM). Increasing ATO concentration resulted in gradual decrease in C.F-GSH level, highest decrease (10.8 μM) being with the maximum of the used ATO concentration (1000 μM) as shown in Table 2 and Figure 3.

Change in C.F-GSH level by ATO at different time fixations: C.F samples having different ATO concentrations were incubated at different time fixations (0-, 30-, 60- and 90-min) and were analyzed for change in GSH level. The pattern of lowering C.F-GSH level seemed to be associated

with the provision of more time as shown in Table 2 and Figure 4.

DISCUSSION

We have studied the effect of arsenic trioxide (ATO) on GSH level in plasma and cytosolic fraction of human venous blood spectrophotometrically. The concentrations of ATO used in the present study ranged from 100 to 1000 μM ATO. The effect of arsenic on the chemical and metabolic status of GSH was studied in terms of determination of concentration of GSH at λ max 412 nm. This λ max (412 nm) is being used for the determination of GSH concentration in samples according to Elman's (1959) method.

Thus the interaction of this metal with glutathione *in vitro* as a model of *in vivo* reaction will establish further scientific data and will strengthen our knowledge about the toxicological

Figure 1. Effect of ATO concentration on plasma GSH content. ■ Plasma control (1 ml 0.9% NaCl/1 ml of blood); ♦ ATO (100 to 1000 μM). Results are the mean $\pm$ SE of 3 experiments of plasma GSH.

Figure 2. Effect of ATO concentration on the plasma GSH content with time incubation period (0 to 90 min); ▲ Plasma control (1 ml 0.9% NaCl/1 ml of blood); ♦ ATO (100 μM); ■ ATO (1000 μM). Results are the mean $\pm$ SE of 3 experiments of plasma GSH

profile of arsenic and the role of GSH in the protection of our body from its harmful effects. According to our

findings, the arsenic in the form of arsenic trioxide (ATO) induced the depletion of GSH in a dose dependent of

Table 2. Effect of different concentrations of arsenic trioxide (0.2 to 2 mM) on the chemical status of glutathione (GSH) with time in cytosolic fraction (arsenic trioxide was injected into whole blood before its separation and incubated for 30 min).

S/N	Concentration of arsenic trioxide (μM)	Average ABS at 0 min	Concentration (μM) of GSH, at 0 min	Average ABS at 30 min	Concentration (μM) of GSH, at 30 min	Average ABS at 60 min	Concentration (μM) of GSH, at 60 min	Average ABS at 90 min	Concentration (μM) of GSH, at 90 min
1	100	0.436	37.6	0.421	36.4	0.404	35.0	0.381	33.1
2	200	0.389	33.8	0.364	31.7	0.347	30.3	0.323	28.4
3	300	0.343	30.0	0.324	28.5	0.301	26.6	0.288	25.5
4	400	0.304	26.8	0.279	24.8	0.261	23.3	0.238	21.4
5	500	0.261	23.3	0.239	21.5	0.221	20.0	0.204	18.6
6	600	0.227	20.5	0.203	18.5	0.181	16.7	0.158	14.8
7	700	0.194	17.8	0.172	16.0	0.149	14.1	0.131	12.6
8	800	0.166	15.5	0.145	13.8	0.128	12.4	0.107	10.7
9	900	0.137	13.1	0.116	11.4	0.102	10.3	0.088	9.1
10	1000	0.109	10.8	0.093	9.5	0.074	8.0	0.058	6.6
CF Control		0.800	67.5	0.800	67.5	0.800	67.5	0.800	67.5

Figure 3. Effect of ATO on cytosolic fraction GSH content; ■ CF control (1 ml 0.9% NaCl/1 ml of blood); ♦ ATO (100 to 1000 μM). Results are the mean $\pm$ SE of 3 experiments of cytosolic fraction GSH.

Figure 4. Effect of ATO on cytosolic fraction GSH content with time incubation period (0 to 90 min); ▲ CF control (1 ml 0.9% NaCl/1 ml of blood); ♦ ATO (100 μM); ■ ATO (1000 μM). Results are the mean ±SE of 3 experiments of cytosolic fraction GSH

manner. The results also show positive correlation between the exposure of glutathione to the given concentrations of ATO and the depletion of GSH as the time passed from 0 to 90 min.

The fact that arsenic trioxide (ATO) has considerable pharmacological effects prompted us to examine the interaction of ATO with glutathione (GSH), the most important intra and extracellular antioxidant in plasma and cytosolic fraction of healthy human volunteers. Furthermore, little information is available on ATO mechanism of action at the molecular level. Our experimental work proposes the molecular mechanism of action of ATO. Our research hypothesis was that ATO directly or indirectly or through enzymatic or non-enzymatic pathway causes modulation in the status of GSH either to glutathione disulfide (GSSG) or formation of arsenic-glutathione (As-SG) complex.

Our results support the hypothesis that incubation and addition of ATO to venous blood, separation of plasma and cytosolic fraction and determination of GSH decreased the concentration of GSH in these components, one of the characteristics of ATO as an oxidant.

The exact mechanism of action of ATO on GSH metabolic status in this study is not known. However the proposed hypothetical mechanism of action of ATO on GSH metabolic status is the formation of As-(SG)₃ complex. This hypothetical mechanism of action and formation of As-(SG)₃ complex is in agreement with our research work, where antibacterial activity of ATO, GSH and ATO and GSH mixture were examined.

The results indicate that antibacterial activity of ATO was very low (almost Zero), GSSG, GSH were high and ATO and GSH mixture was lower than GSH and higher than ATO (data not shown).

These results indicating that antibacterial activity of

GSH and GSSG were the same and GSH and ATO mixture was very low than GSH and GSSG and ATO had almost Zero, leads to the conclusion that ATO had not caused the oxidation of GSH to GSSG but consistent with the reaction taking place at the active site of GSH and that is S-H group.

Such reaction through a mechanism involving the coordination of As in ATO with the S-H group of GSH with the formation of As-(SG)₃ complex is as:

In conclusion, we have examined interaction of ATO with GSH spectrophotometrically and hypothesized the formation of As-(SG)₃ complex, which might have clinical implication.

The data provided in this research work is lacking implementation of statistical analysis, as the blood samples were collected from a single healthy volunteer.

REFERENCES

- Boylard E, Chasseaud LF (1969). The role of glutathione and glutathione S-transferases in mercapturic acid biosynthesis. *Adv. Enzymol. Relat. Areas Mol. Biol.*, 32: 173-219.
- Bremer HJ, Duran M, Kamerling JP (1981). Glutathione In: Bremer HJ, Duran M, Kamerling JP, et al, eds. *Disturbances of Amino Acid Metabolism: Clinical Chemistry and Diagnosis*. Baltimore-Munich: Urban and Schwarzenberg. pp. 80-88
- Cai J, Nelson KC, Wu M (2000). Oxidative damage and protection of the RPE. *Progr. Retinal. Eye Res.*, 19: 205-221.
- Chasseaud LF (1976). Conjugation with glutathione and mercapturic acid excretion, in *Glutathione: Metabolism and Function* (Arias IM and Jakoby WB eds). Raven Press, New York, pp. 77-114.
- Commandeur JNM, Stijntjes GJ, Vermeulen NPE (1995). *Enzymes and*

- transport systems involved in the formation and disposition of glutathione S-conjugates. Role in bioactivation and detoxication mechanisms of xenobiotics. *Pharmacol. Rev.*, 47: 271-330.
- Ellman GL (1959). Tissue sulfhydryl groups. *Arch. Biochem. Biophys.*, 82: 70-77.
- Kidd PM (1997). Glutathione: Systemic protectant against oxidative and free radical damage. *Altern. Med. Rev.*, 1: 155-176.
- Kitchin KT (2001). Recent advances in arsenic carcinogenesis: Modes of action, animal model systems, and methylated arsenic metabolites. *Toxicol. Appl. Pharmacol.*, 172: 249-261.
- Look MP, Rockstroh JK, Rao GS (1997). Serum selenium, plasma glutathione (GSH) and erythrocyte glutathione peroxidase (GSH-Px)-levels in asymptomatic versus symptomatic human immunodeficiency virus-1 (HIV-1)-infection. *Eur. J. Clin. Nutr.*, 51: 266-272.
- Luo J-L, Hammarqvist F, Andersson K (1998). Surgical trauma decreases glutathione synthetic capacity in human skeletal muscle tissue. *Am. J. Physiol.*, 275: 359-365.
- Ratnaike RN (2003). Acute and chronic arsenic toxicity. *Postgrad. Med. J.*, 79: 391-396.
- Spies CD, Reinhart K, Witt I (1994). Influence of N-acetylcysteine on direct indicators of tissue oxygenation in septic shock patients: results from a prospective, randomized, double-blind study. *Crit. Care Med.*, 22: 1738-1746.
- States JC, Reiners JJ, Pounds JG, Kaplan DJ, Beauerle BD (2002). Arsenite disrupts mitosis and induces apoptosis in SV40-transformed human skin fibroblasts. *Toxicol. Appl. Pharmacol.*, 180: 83-91.
- Thomas DJ, Styblo M, Lin S (2001). The cellular metabolism and systemic toxicity of arsenic. *Toxicol. Appl. Pharmacol.*, 176: 127-144.
- Whitcomb DC, Block GD (1994). Association of acetaminophen hepatotoxicity with fasting and ethanol use. *JAMA*, 272: 1845-1850.
- Yamauchi H, Aminaka Y, Yoshida K, Sun G, Pi J (2004). Evaluation of DNA damage in patients with arsenic poisoning: urinary 8-hydroxydeoxyguanine. *Toxicol. Appl. Pharmacol.*, 198: 291-296.
- Zielhuis RL, Wibowo AAE (1984). Standard setting and metal speciation: arsenic, in: J.O. Nriagu (Ed.), *Changing Metal Cycles and Human Health*, Dahlem Konferenzen, Springer, Berlin, pp. 323-344.