THE PROTECTIVE ROLE OF GLUTATHIONE IN SILVER INDUCED TOXICITY IN BLOOD COMPONENTS

HAROON KHAN[1], MUHAMMAD FARID KHAN[1], ASIM-UR-REHMAN[2], SYED UMER JAN[3] AND NASEEM ULLAH[1]

[1]*Department of Pharmaceutical Chemistry,* [2]*Department of Pharmaceutics*
Faculty of Pharmacy, Gomal University, D.I. Khan, Pakistan
[3]*Department of Pharmacy, University of Balochistan, Quetta, Pakistan*

ABSTRACT

Silver Nitrate ($AgNo_3$) effect on the status of reduced glutathione (GSH), the major non-protein thiol in blood components were examined using freshly isolated plasma and cytosolic fraction of whole blood of healthy human volunteer. $AgNo_3$ had significant effects on GSH contents reduction in plasma and cytosolic fraction of blood. Effect or reduction in GSH content was more pronounced or significant at $AgNo_3$ concentration with time intervals (minutes to hours). Depletion of GSH in plasma and cytosolic fraction of blood by the addition of $AgNo_3$ indicates that $AgNo_3$ penetrated the cell membrane and reacted with GSH, depleted its contents mostly due to oxidation of reduced Glutathione (GSH) to glutathione disulphide (GSSG) $2\ GSH \rightarrow GSSG + 2H^+$ or likely that $AgNo_3$ after penetration formed a Silver-Glutathione (Ag-SG) complex. Time dependent effect of $AgNo_3$ on the chemical status of glutathione was discussed *in vitro* in this paper which in principle presents the model of *in vivo* reaction, which will be the precise indirect impression of $AgNo_3$ detoxification resulting by the interaction with GSH.

Keywords: L-Glutathione (GSH), Silver Nitrate ($AgNo_3$), Ag-SG (Silver glutathione Complex), 5, 5- dithiobis 2, 2-nitro benzoic Acid (DTNB).

INTRODUCTION

GSH is found virtually in all cells, and its concentration varies depending upon the number and kind of metabolic needs requiring GSH in these cells and tissues (Friedman, 1973). Blood is also one of the most major cite of GSH content, Erythrocyte contain a large amount of GSH approximately 2-3mM (Hagenfeldt and Larsson, 1928) and leukocyte seven time more (14-21mm) than that of erythrocyte (Hardin *et al*., 1954) and thought to be involved in protecting cells against oxidative damage. The metabolism of thiols in general and GSH in particular can be altered by endogenous and exogenous substances or certain physiological changes and certain selected chemicals and /or compounds have important and toxicological applications. Blood cells rich in GSH have not been given much attention with regards to chemicals or drugs induced toxicity. Also little attention have been paid to capacity of blood to the metabolize xenobiotics although these cells are equipped with a variety of enzymes that are required for biotransformation of chemicals and drugs. GSH serving as a cofactor for some 20 enzymes is often the 1st line of biological defense against tissue injury and the major cellular non-protein thiol. Glutathione is the most sensitive indicator of the cell and it is its ability to resist toxic challenge. Glutathione depletion leads to suicide of cell by a well-known apoptosis process (Slater *et al*., 1995; Duke *et al*., 1996). Although normal Silver concentrations in human tissues are not too much but it can accumulate in the mucous membranes, skin, kidney, liver, gingival, nail and spleen if silver metal is verexposere to these organs (Rosenman *et al*., 1979; Wan *et al*., 1991; Hollinger, 1996; Sue *et al*., 2001). In the liver Silver ion have capacity for GSH (Baldi *et al.*, 1988) and bind there with GSH and transported to bile and cause depletion of GSH require for biochemical pathways. A disease which is called argyria is considered to be a mechanism to diminish the toxicity of Silver ions by confiscating in the tissues as not detrimental Silver-thiol complexes (Venugopal and Luckey, 1978).

A link between Silver and GSH has been demonstrated from several laboratories. A number of chemical, mettalloelements and drugs have toxic and adverse biological effects in the absence of sufficient and efficient defense system. Here GSH plays a role as an efficient defense system in cellular protection against these toxic and adverse effects. These observations with regard to Silver and GSH may serve further to investigate those areas which have been neglected today. In this study GSH content in blood components and Silver salt/complex effect on GSH in blood component will be determined.

MATERIALS AND METHODS

Chemical

Silver nitrate (BDH, Germany), Sodium chloride (Merck), 5, 5 dithiobis, 2 nitro benzoic acid (DTNB) (Sigma), L-glutathione (Fluka), Sodium hydroxide (Fluka AG), Chloroform (Merck), Ethanol (Merck), HCl 35% (Kolchlight), Potassium Dihydrogen phosphate (Merck),

**Corresponding author:* e-mail: suj55@yahoo.com

Disodium edetate (Reidel Dehean AG Sleeze Hannover), Distilled water (double distilled). Water for injection (Elixir Laboratories), UV/Visible Spectrophotometer (Shimadzu), pH meter: Model NOV-210 (NOV scientific company Ltd. Korea), Centrifuge (H-200, kokusan Ensink company Japan), Oven; Memmrt Model U-30, schwabach (Germany), Magnetic stirrer, Hot plate 400, Micropipettes 200 µl, 500µl, 1000µl were used of socorex Swiss (Finland), Sortorius balance, Eppendolf's tubes (Plastic, 10l), Siliconised test tube, Disposable pyrogen syringes (Otsuka, Japan), fresh human volunteer blood (Three healthy volunteers of 20-25 years of age), Disposable rubber gloves (Otsuka, Japan) were used in this research work.

Isolation of plasma and cytosolic fraction of red blood cells from volunteer venous blood

4ml of male healthy volunteer human venous blood using sterile pyrogen free syringes was collected and immediately poured in to 10ml of centrifuge tubes containing 200-400µl 0.5mole/L EDTA-2Na or Heparin solution to prevent clotting of blood. 4ml of (0.2, 0.4, 0.6, 0.8 and1mM) $AgNo_3$ solution was added to whole blood in centrifuge tube. Whole blood was centrifuged for two minutes at 10,000 rpm. Blood tubes were allowed, standing for few seconds. Supernatant pale yellowish plasma was collected and transferred to sample tubes using micropipettes and kept in freezer or on ice for further use. The remaining packed red cells in the sample tube were twice washed with isotonic solution of NaCl 0.9% and again centrifuges at 10,000 rpm for 30 seconds. The supernatant was discarded each time. 0.5ml- 1ml of red cells fraction was pipette out into sample tubes and lysed with 0.5-1ml of distilled water or 5mM/L EDTA-2NA solution at 4 ^oC for 1 hour. Hemoglobin was precipitated by addition of 0.6ml ethanol-chloroform (5:3 V/V) mixtures at 0 ^oC (twice) to 1ml of lysed cells. 1ml of distilled water was added to precipitated hemoglobin lysed cells and centrifuged as before and clear supernatant pale yellowish fraction was collected and transferred to sample tubes and processed further similar to plasma fraction.

GSH determination in plasma

GSH assay was performed by following standard Ellman's method (Ellman's 1959) using DTNB for plasma of blood. Buffer (PH, 7.6, 0.2M) 2.3 ml was taken in cell and/or cuvette of UV/visible spectrophotometer followed addition of 0.2 ml of plasma of blood. DTNB 0.5ml in a buffer was added in to it. After 5 minutes an absorbance of reaction mixture was taken at 412nm using Shimadzu UV/visible spectrophotometer and glutathione level was determined from standard curve of reduced glutathione obtained with 0.2, 0.4, 0.6, 0.8 and 1mM GSH concentration.

Table 1: Effect of Different concentrations of Silver Nitrate ($AgNO_3$) on Glutathione (GSH) level with time in plasma

Blank DTNB solution Absorbance was 0.057 at 412nm							
Glutathione concentration was 26.67 µM in Final Mixture							
S. No.	Conc. of $AgNO_3$	Real absorbance at 0 Minutes	Real absorbance at 30 Minutes	Real absorbance at 60 Minutes	Real absorbance at 120 Minutes	Real absorbance at 150 Minutes	Real absorbance at 180 Minutes
1	6.67µM	0.614	0.574	0.526	0.487	0.448	0.403
2	13.34 µM	0.581	0.546	0.512	0.468	0.428	0.387
3	20.00 µM	0.555	0.516	0.478	0.439	0.406	0.348
4	26.67 µM	0.514	0.467	0.413	0.374	0.331	0.295
5	33.3 µM	0.482	0.441	0.394	0.347	0.304	0.255
Real GSH ABS		0.861	0.859	0.857	0.856	0.854	0.853

R. Absorbance= ABS of Mixture-ABS of DTNB blank

Table 1a: Conversion of absorbances in to Concentration

S. No.	Conc. of $AgNO_3$	Remaining Conc. at 0 Minutes	Remaining Conc. at 30 Minutes	Remaining Conc. at 60 Minutes	Remaining Conc. at 120 Minutes	Remaining Conc. at 150 Minutes	Remaining Conc. at 180 Minutes
1	6.67µM	51.455	47.818	43.455	39.909	36.364	32.273
2	13.34 µM	48.455	45.273	42.182	38.182	34.545	30.818
3	20.00 µM	46.091	42.545	39.091	35.545	32.545	27.273
4	26.67 µM	42.364	38.091	33.182	29.636	25.727	22.455
5	33.3 µM	39.455	35.727	31.455	27.182	23.273	18.818
Blank GSH Conc.		73.909	73.727	73.545	73.455	73.273	73.182

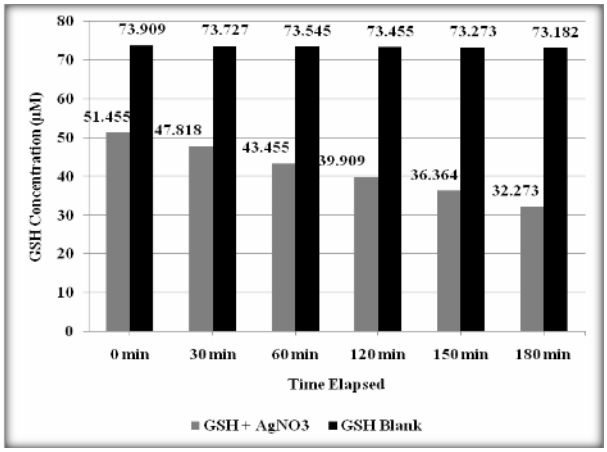

Fig.1a: Effect of $AgNO_3$(6.67µM) on Plasma GSH level.

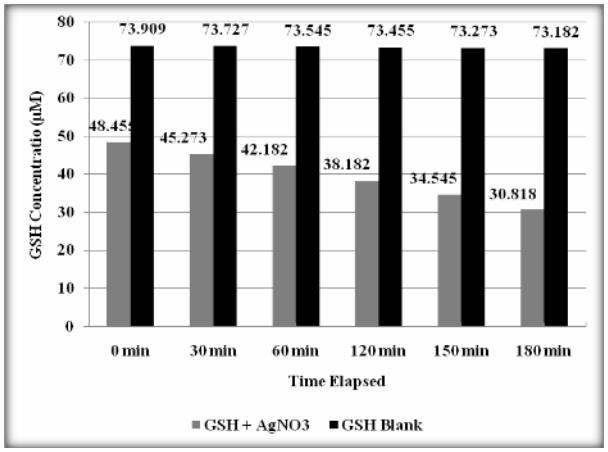

Fig.1b: Effect of $AgNO_3$(13.33µM) on Plasma GSH level.

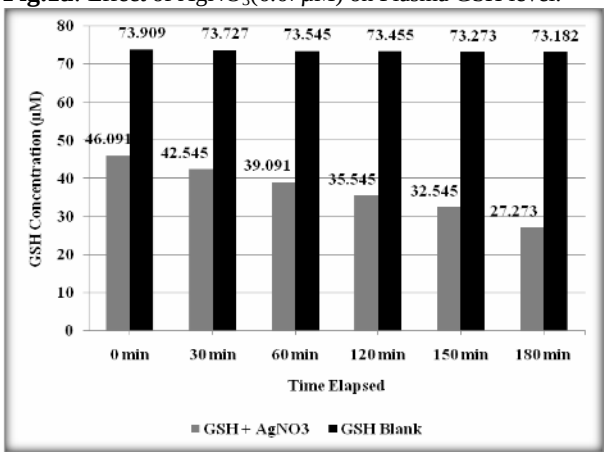

Fig.1c: Effect of $AgNO_3$(20.00µM) on Plasma GSH level.

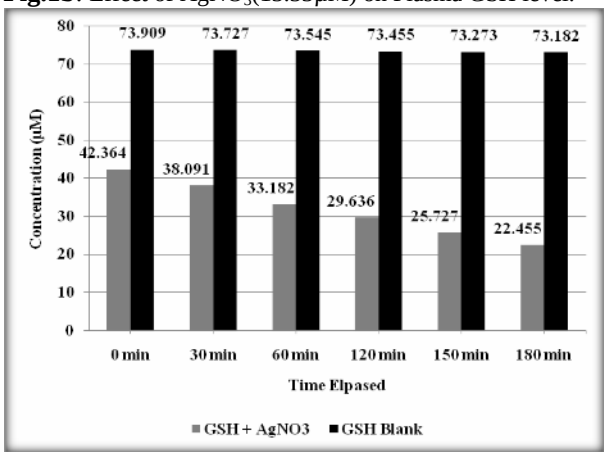

Fig.1d: Effect of $AgNO_3$(26.67µM) on Plasma GSH level.

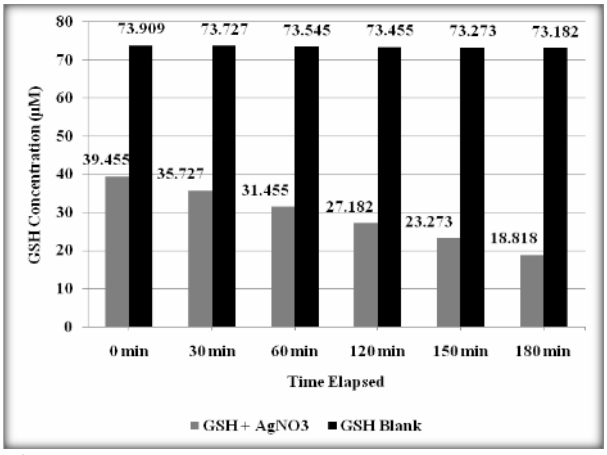

Fig.1e: Effect of $AgNO_3$(33.33µM) on Plasma GSH level.

Determination of GSH in cytosolic fraction

GSH assay was performed by following standard Ellman's method (Ellman's 1959) using DTNB for cytosolic fraction of blood. Buffer (PH, 7.6, 0.2M) 2.3 ml was taken in cell and/or cuvette of UV/visible spectrophotometer followed addition of 0.2 ml of cytosolic fraction of blood. DTNB 0.5ml in a buffer was added in to it. After 5 minutes an absorbance of reaction mixture was taken at 412nm using Shimadzu UV/visible spectrophotometer and glutathione level was determined from standard curve of reduced glutathione obtained with 0.2, 0.4, 0.6, 0.8 and 1mM GSH concentration.

RESULTS

Effect of $AgNo_3$ on plasma thiol/GSH level of human blood

The effect of $AgNO_3$on plasma thiol/GSH level of human blood is given in the in tables 1 and 1a and figs. 1a-5e shows the time dependent effect of $AgNo_3$ on plasma thiol/glutathione.

Effect of $AgNo_3$ on the cytosolic thiol/GSH level of human blood

$AgNO_3$ was added to whole blood of human volunteers. Reduced GSH was measured at time intervals. Decreased concentration was observed at time intervals as compared to control as shown in table 2, and figs. 2a-2e.

Table 2: Effect of Different Dilutions of Silver Nitrate ($AgNO_3$) on (GSH) level with time in cytosolic fraction of blood

Blank DTNB solution Absorbance was 0.057 at 412nm							
Glutathione Concentration was 26.67 µM in Final Mixture							
S. No.	Conc. of $AgNO_3$	Real absorbance at 0 Minutes	Real absorbance at 30 Minutes	Real absorbance at 60 Minutes	Real absorbance at 120 Minutes	Real absorbance at 150 Minutes	Real absorbance at 180 Minutes
1	6.67µM	0.522	0.485	0.426	0.394	0.368	0.336
2	13.34 µM	0.479	0.444	0.402	0.374	0.338	0.299
3	20.00 µM	0.448	0.410	0.385	0.359	0.302	0.258
4	26.67 µM	0.412	0.378	0.329	0.297	0.258	0.226
5	33.3 µM	0.364	0.319	0.287	0.236	0.185	0.142
Real GSH ABS		0.624	0.621	0.623	0.623	0.624	0.622

R. Absorbance= ABS of Mixture-ABS of DTNB blank

Table 2a: Conversion of Absorbance in to Concentration

S. No.	Conc. of $AgNO_3$	Remaining Conc. at 0 Minutes	Remaining Conc. at 30 Minutes	Remaining Conc. at 60 Minutes	Remaining Conc. at 120 Minutes	Remaining Conc. at 150 Minutes	Remaining Conc. at 180 Minutes
1	6.67µM	43.091	39.727	34.364	31.455	29.091	26.182
2	13.34 µM	39.182	36.000	32.182	29.636	26.364	22.818
3	20.00 µM	36.364	32.909	30.636	28.273	23.091	19.091
4	26.67 µM	33.091	30.000	25.545	22.636	19.091	16.182
5	33.3 µM	28.727	24.636	21.727	17.091	12.455	8.545
Blank GSH Conc.		52.364	52.091	52.273	52.273	52.364	52.182

DISCUSSION

In the present study different dilutions (6.67, 13.34, 20.0, 26.67, 33.34 µM) of $AgNO_3$ were injected to plasma and cytosolic fraction of human blood in separate procedures. GSH level continued to deplete as the time elapsed. The observations were recorded at different time intervals from 0 to 180 minutes. The highest depletion was ascribed to the longest period. Similarly the highest decrease in GSH level was observed with the highest concentration (33.34 µM) of $AgNo_3$. This study shows metalloelement (Silver) has concentration and time wise decreased the level of GSH in plasma and cytosolic fraction**.** Glutathione has a reducing capacity for exogenous compounds like Silver and converts itself to oxidized state which is a disulphide (GSSG). These results for $AgNO_3$ conform to our previous findings about metals like Lithium, Mercury, Copper, (Khan *et al*., 2010; Khan *et al*., 2009; Khan *et al*., 2008; Badshah *et al*., 2002), All of which shows decreased GSH level in the same manner.

$$GSH + AgNo_3 \rightarrow Ag\text{-}GS$$

We can consider oxidation of GSH induced by Ag Compounds through the ability of GSH to cleave disulphide (GSSG) (Willson *et al*., 2003; Szagewsi and Whitesides 2004). The study has shown that GSH behaves as a normal nucleophile in attack on disulphide (Willson *et al*., 2003). The GSH attack on sulphitolysis of disulphide (GSSG) is preferential because of its availability ion principle. The cleavage of disulphide (GSSG) by nucleophiles (e.g. OH^-) is catalyzed electrophilicaly by Ag^+. The same results are confirmed by mercury ion (Hg^{+2}) which served disulphide (GSSG) cleavage (Dougals and Shinkei, 2005).

So the oxidation of GSH to GSSG brought about by Ag compounds (Dougals and Shinkei, 2005) can definitely be displayed as under, which attests the depletion of GSH level by Ag compounds.

$$RAgAgR + 2GSH \rightarrow 2R\ AgH + GSSG$$

Glutathione is well known to be the most prevalent non protein thiol relatively in higher concentration intracellularly, and is actively involved in detoxification and excretion of heavy metals through marcapturic acid path way (William *et al*., 2007), rendering the metal to harmless complexes most often with thiols (Metal-Thiol Complex).

Our study indicates decrease GSH level in plasma (table 1a) and cytosolic fraction (table 2a) due to Silver. The decrease in GSH level in cytosolic fraction due to $AgNo_3$ is greater as compared with plasma. This is because that metal penetration through cell membrane of components of cytosolic fraction is higher, where it confronts with the rich intracellular concentration of GSH (Kosower and Kosower, 2005).

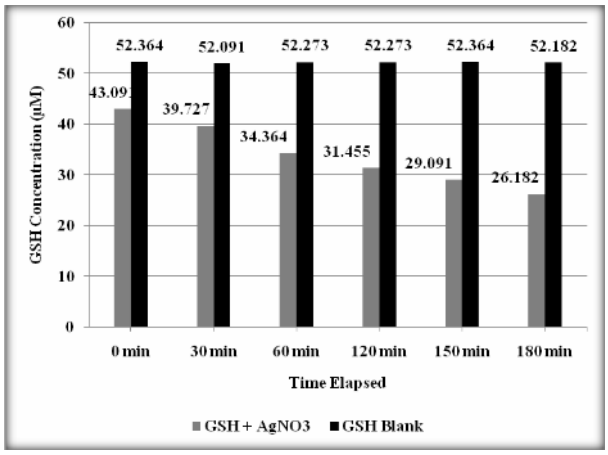

Fig.2a: Effect of $AgNO_3$(6.67µM) on C.F GSH level.

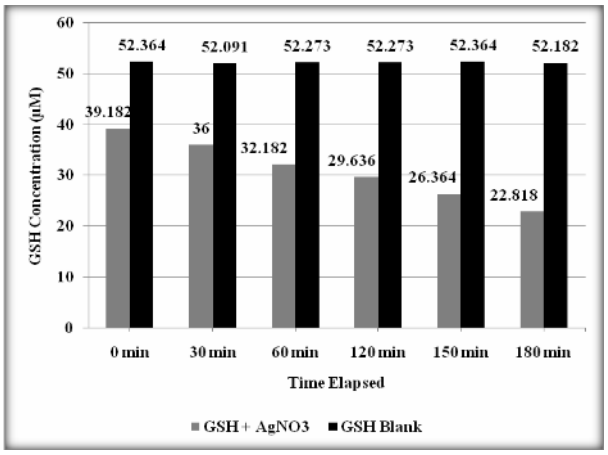

Fig.2b: Effect of $AgNO_3$(13.34µM) on C.F GSH level.

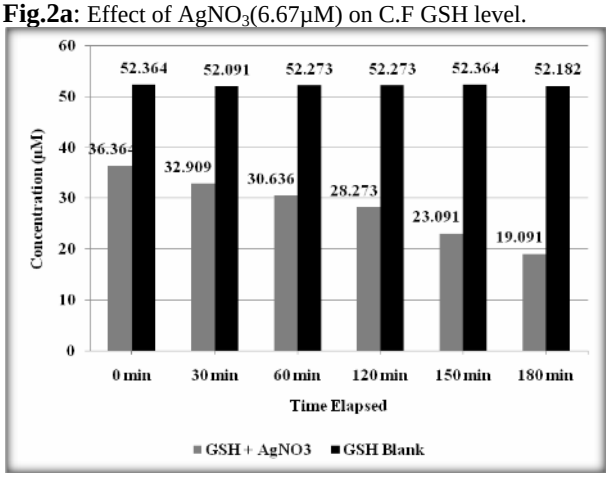

Fig.2c: Effect of $AgNO_3$(20.00µM) on C.F GSH level.

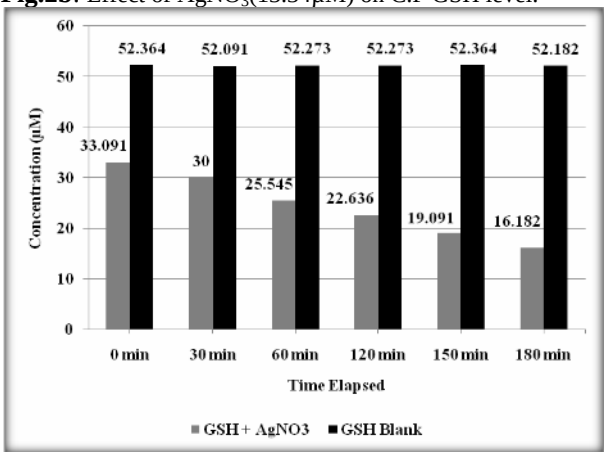

Fig.2d: Effect of $AgNO_3$(26.67µM) on C.F GSH level.

The importance of interaction of metalloelements including $AgNo_3$ with GSH as a biomarker of detoxification may guide biochemical scientist to take account of metal salt/complexes and metal/ drug complexes for implementation into clinical settings.

CONCLUSION

The study revealed that glutathione protects the body from metal toxicity by forming complex with silver which can be helpful in detoxification of silver in the body. The results shown in this experimental work gives impact that $AgNo_3$ if accumulated below 35 µM can effectively be detoxified by GSH.

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