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Full Length Research Paper

Oxidation of glutathione (GSH) in blood plasma due to oxidative stressors: A case study of silver

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The widespread use of silver went out of fashion with the development of modern antibiotics. However, recently, there has been renewed interest in silver as a broad spectrum antimicrobial. The toxicity of silver to human cells is considerably lower than that of bacteria. Silver itself is nontoxic, but most silver salts are, and some of them are to be carcinogenic and may lead to death. That is why the effect of silver on glutathione (GSH) level is interesting to study. The following study was carried out to find out if the effect on GSH level in plasma was studied spectrophotometrically using Ellman's method. Two parameters concentration and time effect of silver was used to determined GSH level in plasma. This study shows that when the concentration of silver was increased and time of incubation extended, a depletion of GSH level in plasma was found. The decline in the GSH level was concentration and time of interaction dependent; due to oxidation of reduced GSH to corresponding oxidize GSH (GSSG) or due to the formation of silver GSH complex (Ag-GS). From this finding, we can conclude that GSH plays an important role in the detoxification and management of silver induced complications and toxicity.

Key words: Silver (Ag), 5, 5-dithiobis, 2, nitrobenzoic acid (DTNB), Ellman's method, glutathione (GSH).

INTRODUCTION

Silver, arsenic, mercury, cadmium and copper metals possess a high affinity for thiol groups (Khan et al., 2010a, 2009, 2012; Ullah et al., 2012). In the cell, glutathione (GSH) is the most abundant low-molecular-weight thiol containing molecule. The roles of GSH in metabolic regulation and cellular function have been extensively reviewed. (Khan et al., 2010b; Larsson et al., 1987; Meister, 1988; Meister and Andersson, 1983). Toxic responses of majority of metals are produced due to interaction of metals with GSH metabolism. Protection of cells against metal toxicity depends on the complexation of GSH with several metals and may thus function in the cell, because GSH forms complexes with several metals.

Depletion of GSH potentiates metal toxicity (Khan et al., 2010a; Cartana et al., 1992; Kang and Enger, 1988). Inside cells, mainly GSH is present in its reduced (electron-rich) GSH form. The oxidized (electron-poor) (GSSG) form of GSH in the healthy cell rarely exceeds 10% of the total cell GSH (Khan et al., 2008, 2009). GSH inside the cell is considered to be the most sensitive indicator of the cell's overall health, and of its ability to resist toxic challenge. GSH depletion in cell can trigger suicide of the cell by a process known as apoptosis (Khan et al., 2009, 2010a, 2011). Although, normal silver concentrations in human tissues are not too much if they are overexposed, silver can accumulate in the corneas, kidneys, liver, skin, gingiva, mucous membranes, spleen and nails (Sue et al., 2001; Hollinger, 1996; Wan et al., 1991; Khan et el., 2008, 2011). In the liver, silver ions have a high affinity for the GSH /thiol groups (Khan et al., 2008, 2011; Baldi et al., 1988) and have been shown to

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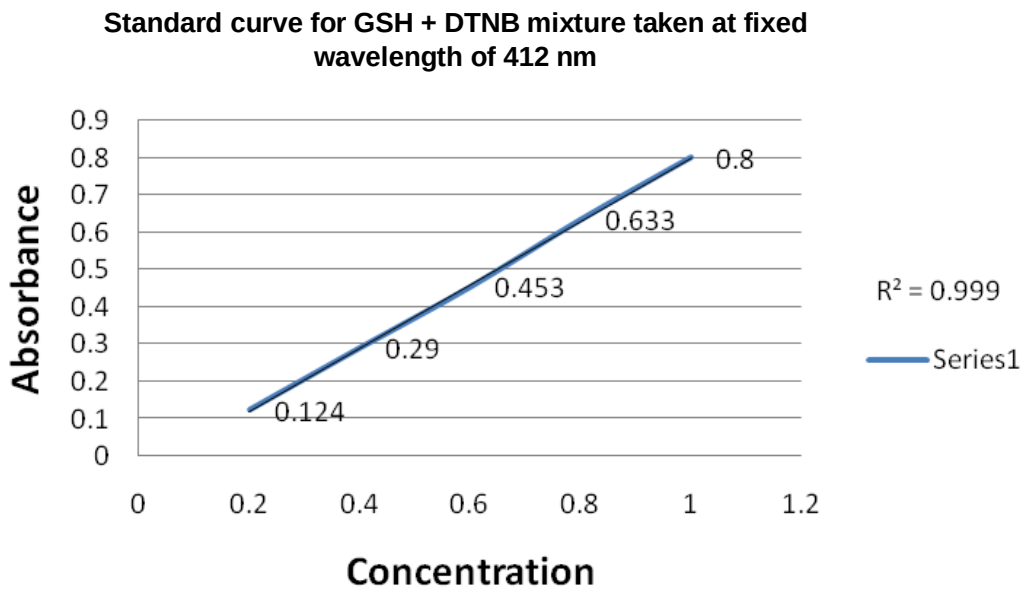

Figure 1. GSH standard curve taken at fixed wavelength of 412 nm.

bind to reduced GSH and be transported into the bile, thus depleting the amount of reduced GSH available for biochemical pathways. The role of reduced form of GSH is very important. It plays a role in normal function of red blood cells, maintaining proper structure and eliminating organic peroxides (Baldi et al., 1988). Very little information is there that show possible toxic effects when silver is deposited in different types of organs and tissues. A disease which is called argyria is considered to be a mechanism to diminish the toxicity of silver by sequestering it in the tissues as harmless silver-protein complexes or silver sulfide (Bishara et al., 2007).

MATERIALS AND METHODS

Sodium hydroxide (Fluka AG), L. GSH (Fluka), 5,5-dithiobis,2-nitrobenzoic acid (DTNB) (Sigma), potassium dihydrogen phosphate (Merck), 35% HCl (Kolchlight), silver nitrate (BDH, Germany), sodium chloride (Merck), and disodium edetate (Riedel Dehean AG sleeze hannover). Chloroform (Merck), ethanol (Merck), and water for injection (Elixir Laboratories), distilled water (Double distilled). UV/Visible 1601 spectrophotometer (Shimadzu). pH Meter: Model NOV-210 (Nova Scientific Company Ltd., Korea), Oven: Memmert Model U-30, 854 Schwabach (Germany). Magnetic stirrer, hot plate 400 (England). Micropipettes of 200, 500 and 1000 µl were used (socorex swiss, Finaland), Sortorius balance, Centrifuge (H-200, Kokusan Ensink Company, Japan), Eppendolf's tubes (Plastic, 10l), Siliconized glass test tubes, sterile pyrogen free disposable syringes (B.D), fresh human volunteer blood (Three healthy volunteers of 20 to 25 years of age), disposable rubber gloves (Otsuka, Japan), were used in this research work.

Isolation of plasma

In the isolation of plasma, 2 ml of blood was taken from the vein of volunteer by using syringe previously dipped with 0.5 M sodium edetate solution. The blood was softly mixed in the syringe, and 1.8 ml of the blood was transferred to Eppendolf's test tube (2.0 ml), and was centrifuged softly for 10,000 rpm for 2 min. Red blood cell l of 5 mMµ was precipitated down. Supernatant of 0.5 ml was taken and was softly mixed with 50 sodium edetate and was placed in refrigerator till use.

Determination of GSH in plasma

The assay of GSH with DTNB was performed by following followed a standard Ellman's method (1959). For plasma of blood, 2.3 ml of potassium phosphate [0.2 M, pH 7.6] buffer was taken in the cell and/or cuvette, followed by the addition of 0.2 ml aqueous solution or plasma of blood. To the plasma solution, 0.5 ml [DTNB] (0.001 M) in a buffer was added. An absorbance of reaction product in the cuvette was read after 5 min at 412 nm using Shimadzu 1601 UV/Visible double bean spectrophotometer and GSH level was determined, from standard curve of reduced GSH obtained with 0.2, 0.4, 0.6, 0.8 and 1 mM GSH concentration.

Standard curve for GSH (Ellman's method)

Phosphate buffer (2.3 ml) of pH 7.6 was added to 200 µl of 0.2, 0.4, 0.6, 0.8, and 1 mM solutions of GSH, followed by the addition of 0.5 ml of 1 mM DTNB stock solution. For 5 min at 30°C in oven, the mixtures were placed after it has been thoroughly shaken. After 5 min, absorbance was taken at fixed wavelength of 412 nm. GSH blank was also prepared in which GSH was omitted. Change of absorbance versus final concentration of GSH in the mixture was determined by constructed standard curve. Using linear regression analysis, a straight line was drawn. Correlation coefficient of the plot is 0. 999. Figure 1 shows the standard curve.

Silver nitrate different concentrations effect on GSH level in plasma

One microliter of different concentrations of 0.02, 0.04, 0.06, 0.08,

Table 1. Different concentrations of silver nitrate effect on GSH level in plasma.

S/N	Conc. used of $AgNo_3$ (mM)	Final conc. of $AgNo_3$ in mixture (µM)	1st reading	2nd reading	3rd reading	Average of the 3 readings	Real absorbance*	Real absorbance for blank plasma
1	0.02	6.67	0.410	0.396	0.391	0.399	0.341	0.484
2	0.04	13.33	0.385	0.371	0.366	0.374	0.316	0.474
3	0.06	20.00	0.375	0.361	0.356	0.364	0.306	0.463
4	0.08	26.67	0.349	0.335	0.330	0.338	0.280	0.463
5	0.1	33.33	0.310	0.296	0.291	0.299	0.241	0.472

Absorbance of mixture - absorbance of DTNB blank solution = *Real absorbance. Absorbance of DTNB blank solution was 0.060 ABS at 412 nm.

Table 2. Time dependent effect of silver nitrate on GSH level in plasma.

S/N	Time (min)	1st reading	2nd reading	3rd reading	Average of 3 readings	Real absorbance*	GSH blank ABS	Real absorbance for GSH blank
1	0	0.310	0.298	0.316	0.308	0.250	0.550	0.492
2	30	0.270	0.258	0.276	0.268	0.210	0.540	0.482
3	60	0.250	0.240	0.257	0.249	0.191	0.545	0.487
4	90	0.245	0.233	0.251	0.243	0.185	0.538	0.480
5	120	0.235	0.229	0.244	0.236	0.178	0.543	0.485
6	150	0.210	0.201	0.218	0.210	0.152	0.530	0.472

Absorbance of mixture - absorbance of DTNB blank solution= *****Real absorbance. DTNB blank solution absorbance was 0.060 at 412 nm. Final concentration of silver nitrate was 33.33 µM in final mixture.

and 0.1 mM solution of silver nitrate were added separately to 1 ml of plasma taken in five separate test tubes and were then shook (Table 1). Silver nitrate of 0.2 ml plus plasma mixture separated in five tubes were prepared from each previously made five tubes diluted with 2.3 ml of phosphate buffer (pH 7.6) and was added 0.5 ml of 1 mM DTNB stock solution. By taking 1 ml of plasma in a test tube and diluted with 1 ml of phosphate buffer pH 7.6 to prepare a control for plasma. A well known Ellman's method (Khan et al., 2012; Shah et al., 2012; Ahmad et al., 2012) was used to see the silver nitrate effect on GSH level in plasma in terms of the determination of the concentration of GSH in the mixtures. The concentrations of GSH were determined from the GSH standard curve.

Time dependent effect of silver nitrate on GSH level in plasma

One milliliter of solution of silver (0.1 mM) was added to 1 ml of plasma taken in a test tube and was shook. The final concentration of silver nitrate was 0.5 mM. Five separate tubes of 0.2 ml silver nitrate plus plasma mixture were prepared from each previously made five tubes and was added 0.5 ml of 1 mM DTNB stock solution which was diluted with phosphate buffer of 2.3 ml (pH 7.6). Plasma of 1 ml was taken in a test tube and was diluted with 1 ml phosphate buffer (pH 7.6) to prepare a control for the plasma. At 0, 30, 60, 90, 120, and 150 min, the absorbance was taken after preparing the mixture (1 ml of plasma plus 1 ml of silver nitrate) (Table 2). The concentrations of GSH in plasma were determined from the GSH standard curve.

Statistical analysis

Results are presented as a mean ± standard deviation (SD). Statistical significance and difference from control (GSH in plasma) and test values (GSH in plasma + Silver nitrate) were evaluated by Student's paired t- test. Correlation co-efficient was used to describe the effect of on variable on the other by Pearson's correlation test. $P < 0.05$ was considered as the level of significance. Statistical calculations are given in Tables 5 and 6.

RESULTS AND DISCUSSION

Silver nitrate effect on GSH level in plasma

GSH 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 (Khan et al., 2011), rendering the metal to harmless complexes most often with thiols (Metal-Thiol Complex). The effect of silver nitrate on GSH level present in the plasma was determined in term of determining the concentration of GSH Concentration of GSH present in plasma was decreased due to silver nitrate. Silver nitrate different concentrations causes a gradual decrease in the concentration of GSH in the plasma as the concentration of silver increased as shown in Figure 2 and Table 3. Time dependent effect of silver nitrate on GSH level was also determined and it was noted that the concentration of GSH was depleted gradually as the time passes from 0 to 150 min as shown in Figure 3 and Table 4. The reason

Table 3. Concentration calculation for GSH after reaction with silver nitrate by Ellman's method.

S/N	Real absorbance	Remained concentrations of GSH (µM) in plasma
1	0.341	29.844
2	0.316	27.795
3	0.306	26.975
4	0.280	24.844
5	0.241	21.648

Table 4. Concentration calculation for GSH after reaction with silver nitrate by Ellman's method with time.

S/N	Real absorbance	Remained concentration of GSH (µM) in plasma	Absorbance of blank plasma	Conc. of GSH in plasma blank (mM)
1	0.250	22.385	0.492	0.637
2	0.210	19.107	0.482	0.625
3	0.191	17.549	0.487	0.631
4	0.185	17.057	0.480	0.623
5	0.178	16.484	0.485	0.629
6	0.152	14.352	0.472	0.613

Table 5. Statistical analysis of the effect of silver nitrate on GSH chemical status in plasma (Plasma) of blood.

Statistics of paired samples						
		Mean	**SD**	**SEM**	**N**	
Pair	Silver + Plasma	0.2968	0.0374	0.007	5	-
	Blank plasma	0.471	0.009	0.002	5	-

Correlations of paired samples				
		Pearson's correlation	**N**	
Pair	Silver + Plasma Blank plasma	0.477	5	-

Test for paired samples									
		Paired differences					**t**	**df**	**(1-Tail) t-Critical**
		Mean	**SD**	**SEM**	**Difference of 95% CI**				
					Lower limit	**Upper limit**			
Pair	Silver + Plasma Blank plasma	0.2968	0.0374	0.000	0.263	0.329	-11.21	5	2.131

SEM, Standard error mean; SD, standard error.

for the decrease concentration of GSH in plasma is that silver nitrate has high affinity for the GSH present in the plasma of blood, because silver nitrate binds to sulfhydryl groups of GSH. Depletion of the reduced form of GSH in the plasma of blood is due to the affinity of silver nitrate for GSH. This result confirms these findings (Quig, 1998; Stohs and Bagchi, 1993; Hultberg et al., 2001) that reported that long term exposure to metal, such as silver depletes the level of reduced GSH in the plasma of blood due to high affinity of silver nitrate to the sulfhydryl group of GSH. GSH 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 gallium, antimony, arsenic, and mercury (Ahmad et al., 2012; Shah et al., 2012; Khan et al., 2012; Badshah et al., 2002), all of which shows decreased GSH level in the same manner.

Table 6. Statistical analysis of effect of Silver nitrate on GSH chemical status in plasma (Plasma) of blood with time.

Statistics of paired samples						
		Mean	SD	SEM	N	
Pair	Silver + Plasma	0.194	0.033	0.006	6	-
	Blank plasma	0.483	0.006	0.001	6	-

Correlations of paired samples				
		Pearson's correlation	N	
Pair	Silver + Plasma Blank plasma	0.825	6	-

Test for paired samples									
		Paired differences					t	df	(1-Tail) t-Critical
		Mean	SD	SEM	Difference of 95% CI				
					Lower limit	Upper limit			
Pair	Silver + Plasma Blank plasma	0.194	0.033	0.000	0.22	0.168	-25.4	5	2.015

SEM: standard error mean; SD: standard error.

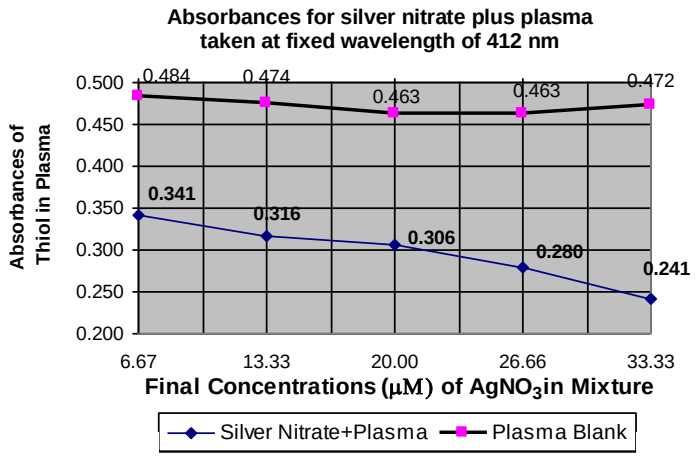

Figure 2. Curves for plasma thiol control level and $AgNo_3$ affected plasma thiol level.

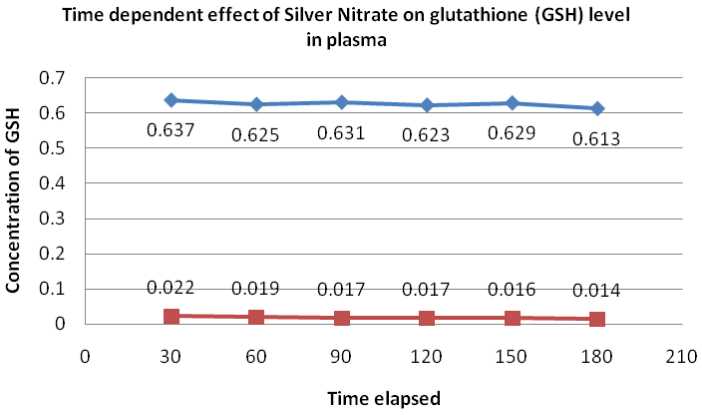

Figure 3. Time curve for plasma thiol control level (▄) and $AgNo_3$ affected plasma thiol level (▄).

Conclusion

From these findings, we concluded that silver gradually decreased the concentration of GSH in plasma due to different concentration of silver nitrate. Silver nitrate effect on GSH level was also studied for the time dependency and it was noted that when the time of incubation was extended from 0 min interval of time to 150 min, the concentration of GSH declined in plasma. So, silver decreases the concentration of GSH. In this way, we can prepare antidote which can be used in the detoxification of toxic metals which are harmful to the human body.

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