CONCENTRATION AND TIME DEPENDENT EFFECT OF SILVER METAL ON GLUTATHIONE LEVEL IN RED BLOOD CELL (CYTOSOLIC FRACTION) OF HUMAN BLOOD

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ABSTRACT

The antibacterial activity of silver has long been known and has found a variety of applications because its toxicity to human cells is considerably lower than to bacteria. Silver itself is not toxic but most silver salts are, and some of them may be carcinogenic. Thus it is interesting to study the effect of Silver on the glutathione (GSH). The effect of silver on the chemical status of the Glutathione (GSH) in cytosolic fraction has been studied using U.V Spectrophotometer by using Ellman's method. The effect of silver on the chemical status of glutathione (GSH) was checked in cytosolic fraction for concentration and time dependent effects. There was found a profound effect on decreasing the concentration of glutathione (GSH) in cytosolic fraction as the concentration is increased and time has passed. The decrease in the glutathione level was concentration and time of interaction dependent, probably due to oxidation of GSH to corresponding disulphide (GSSG).In this paper the effect of silver metal on thiol/GSH level was discussed *in vitro*, which in principle may present a model of *in vivo* reaction.

KEYWORD:

Silver,Glutathione (GSH), Red Blood Cell (Cytosolic Fraction), Ellman's method 5, 5-Dithiobis, 2-Nitrobenzoic Acid (DTNB)

INTRODUCTION:

Glutathione (GSH) is widely distributed throughout in nature, and is found in animal tissues, plants and micro-organisms [Verjee and Behal, 1998) due to physiological significance. GSH is found in virtually all mammalian cells in relatively high concentration i.e.0.1-10mM [Meister, 1973; Meister and Tate, 1976; Sies and Wendel, 1978; Kisower and Kisower, 1978). Glutathione (GSH) is the most prevalent cellular Thiol and the most abundant low molecular weight polypeptide. In many cells GSH accounts for more than 90% of the total non-protein sulfur Meister, 1988). The concentration of GSH in tissues is varied depending on the need, number and kind of the metabolic processes requiring Glutathione (GSH) in these tissues. Most tissues contain GSH concentration being as 15mM in the lens[5] -10mM in the liver [Sies and Wendel 1978). Liver is quantitatively the most important site of the Glutathione (GSH) synthesis. It plays a central role in providing Glutathione (GSH) for cellular protection against reactive intermediate & pollutant Xenobiotics (Chasseaud, 1979).

The reduced glutathione molecule consists of three amino acids - Glutamic acid, Cysteine, and Glycine - covalently joined end-to-end. The sulfhydryl (-SH) group, which gives the molecule its electron-donating character, comes from the cysteine residue.Glutathione is present inside cells mainly in its reduced (electron-rich, antioxidant) GSH form. In the healthy cell GSSG, the oxidized (electron-poor) form, rarely exceeds 10 percent of total cell Glutathione (GSH) (Kosower and Kosower, 1978). Intracellular GSH status appears to be a sensitive indicator of the cell's overall health and of its ability to resist toxic challenge. Experimental GSH depletion can trigger suicide of the cell by a process known as apoptosis (Duke *et al*., 1996; Slater *et al*., 1995).

Silver has affinity for the Glutathione (GSH) present in aqueous phases of blood. This affinity is mainly formed between metal and sulfhydryl groups of proteins(Quig, 1998).This affinitiy can cause a depletion of the reduced form of Glutathione in the blood ,but with the depletion of the Glutathione

(GSH),GSH synthesizing systems start making more GSH from cysteine via the γ-glutamyl cycle but if GSH is usually not effectively supplied, however, if GSH depletion continues because of chronic metal exposure (Quig, 1998; Hultberg *et al.*, 2001 Stohs and Bagchi,1993) then the pharmacological benefits of the metal being used for the help of body defenses can be harmful in nature to the body defense system . The following study makes a design to see the effects of Silver, in respect of concentration and time, on glutathione level in cytosolic fraction.

MATERIALS AND METHODS

Materials

L.glutathione (GSH) was purchase from (Fluka) 5,5-Dithiobis, 2-Nitrobenzoic Acid (DTNB) was from (Sigma) chemical Co, Silver Nitrate was obtained from (Across Belgium).All other reagent were of the highest purity commercially available. U.V 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 200 µl, 500 µl, 1000 µl were used of Socorex Swiss (Finland), Sortorius Balance, , Disposable Rubber Gloves, were also used in this research work .

Isolation of Cytosolic Fraction

0.5ml red cell fraction was taken left, after isolation of plasma and washed 2-3 times with 1ml of 0.9% NaCl Stock Solution (Mixed and centrifuge for 5 minutes and discard Supernatant).then 0.5ml of washed red cell fraction was taken and added 0.5ml of 5 mM sodium edetate solution. Mixed it and stored in refrigerator for 1hr.

0.6ml of cold chloroform: Ethanol (3:5) mixture was added and mixed thoroughly to precipitate hemoglobin. Mixed it and add 0.1ml of water. Then centrifuged it for 10 minutes at 10000-12000 rpm. Then supernatant (pale yellow)-(Lysate or cytosolic fraction) was taken and kept it in refrigerator until used. (Ellman, 1959)

METHODS

Standard Curve for Glutathione

200µl of 0.2, 0.4, 0.6, 0.8 and 1mM solutions of glutathione was added to 2.3ml of phosphate buffer (pH 7.6), followed by the addition of 0.5ml of 1mM DTNB stock solution. The mixtures were shaken thoroughly and incubated for 5 minutes at 30°C. Absorbances were taken after 5 minutes at fixed wavelength of 412nm.

Blank was prepared in which GSH was omitted. Standard curve was constructed by plotting the change of absorbance versus final concentration of GSH in the mixture. Straight line was drawn by using linear regression analysis. The correlation coefficient of plot was 0.9984. Standard curve was obtained as shown in figure

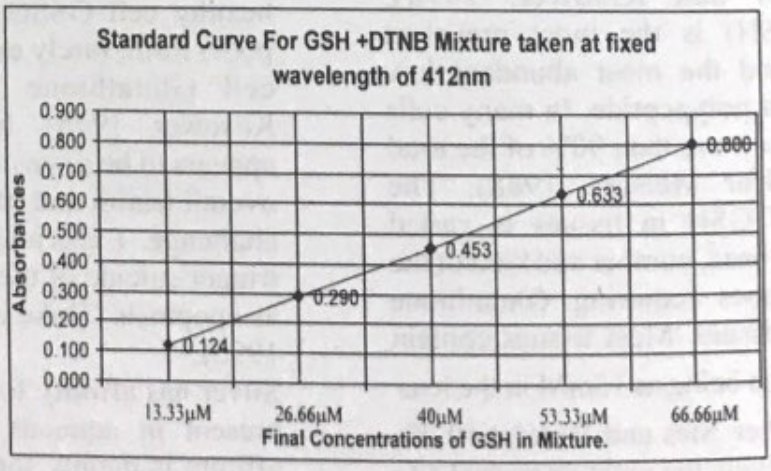

Figure 1- Standard Curve for Glutathione (GSH) + DTNB Mixture taken at fixed wavelength of 412nm

Effect of different concentrations of Silver Nitrate on Glutathione Level in Cytosolic Fraction

To 1ml of cytosolic fraction taken in five separate test tubes, 1ml of different concentrations of 0.02, 0.04, 0.06, 0.08 and 0.1mM solution of silver nitrate were added separately and shaked. Five separate tubes were prepared with 0.2ml silver nitrate plus cytosolic fraction mixture from each previously made five tubes diluted with 2.3ml of phosphate Buffer pH 7.6 and added 0.5ml of 1mM DTNB stock solution. A control for cytosolic fraction was also prepared by taking 1ml of cytosolic fraction in a test tube and diluted with 1ml of phosphate buffer (pH 7.6). The effect of silver nitrate on the chemical status of GSH in cytosolic fraction was studied in terms of determination of concentration of GSH in mixtures by a well known Ellman's method, as mentioned in standard curve for GSH. The concentrations of GSH were determined from the GSH standard curve.

Table 1-Effect of different concentrations of silver nitrate on Glutathione (GSH) level in Cytosolic Fraction.

Absorbance of 5,5-Dithiobis,2-Nitrobenzoic Acid (DTNB) blank Solution was0.060 at 412nm

S#No	Conc. Used of AgNo3	Final Conc. of silver nitrate in Mixture	1st ABS	2nd ABS	3rd ABS	Average of 3 Readings	Real absorbance*	Real Absorbance for Cytosolic Fraction Blank
1	0.02mM	6.67µM	0.120	0.124	0.119	0.121	0.063	0.098
2	0.04mM	13.33µM	0.110	0.115	0.110	0.112	0.054	0.100
3	0.06mM	20.00µM	0.101	0.105	0.100	0.102	0.044	0.089
4	0.08mM	26.67µM	0.093	0.094	0.091	0.093	0.035	0.101
5	0.1mM	33.33µM	0.086	0.088	0.084	0.086	0.028	0.094

** Real Absorbance = Absorbance of Mixture - Absorbance of DTNB blank Solution.*

Effect of Silver Nitrate on Glutathione (GSH) Level in Cytosolic Fraction with time

To 1ml of cytosolic fraction taken in a test tube, 1ml of 0.1mM solution of silver nitrate was added and shaked. The final concentration of silver nitrate was 0.5 mM. A test tube with 0.2ml silver nitrate plus cytosolic fraction mixture was prepared from previously made test tube diluted with 2.3ml of phosphate (buffer pH 7.6) and added 0.5ml of 1mM DTNB stock solution. The final concentration of silver nitrate was 0.03333 mM .A control for cytosolic fraction was also prepared by taking 1ml of cytosolic fraction in a test tube and diluted with 1ml of phosphate buffer (pH 7.6).

The effect of silver nitrate on the chemical status of GSH in cytosolic fraction was studied in terms of determination of concentration of GSH in mixtures by a well known Ellman's method, as mentioned in standard curve for GSH. The absorbances were read at 0, 30, 60, 90, 120; 150 minutes after preparing mixture (1ml of cytosolic fraction plus 1ml of silver nitrate. The concentrations of GSH in cytosolic fraction were determined from the GSH standard curve.

RESULTS

Effect of Silver on the Chemical Status of Glutathione (GSH) in Cytosolic Fraction

Effect of silver metal on the chemical status of glutathione present in cytosolic fraction was studied in term of determination of concentration of GSH.

Silver metal caused a decrease in the concentration of GSH present in cytosolic fraction. Different concentrations of silver cause a gradual decrease in the concentration of GSH in plasma as the concentration of metal increased as shown figure 2 and table 3.Effect of silver on the chemical status of GSH was also studied for the time dependency and noted that the concentration of GSH was gradually decreased as the time passes from (0 minute interval of time to 150 minutes) as shown figure 3 and table 4.

Table 2 Effect of Silver Nitrate on Glutathione (GSH) level in Cytosolic Fraction with time

Absorbance of 5,5-Dithiobis,2-Nitrobenzoic Acid (DTNB) blank Solution was 0.060 at 412nm

Final Concentration of Silver Nitrate was 33.33μM in Final Mixture

S#No	Time Interval	1st ABS	2nd ABS	3rd ABS	Average of 3 Readings	Real absorbance*	GSH Blank ABS	Real Absorbance for GSH Blank
1	0 min	0.074	0.062	0.055	0.064	0.008	0.179	0.121
2	30 min	0.070	0.058	0.051	0.060	0.004	0.176	0.118
3	60 min	0.068	0.056	0.049	0.058	0.002	0.174	0.116
4	90 min	0.065	0.053	0.046	0.055	-0.001	0.173	0.115
5	120 min	0.050	0.038	0.031	0.040	-0.016	0.170	0.112
6	150 min	0.051	0.039	0.032	0.041	-0.015	0.167	0.109

** Real Absorbance = Absorbance of Mixture - Absorbance of DTNB blank Solution*

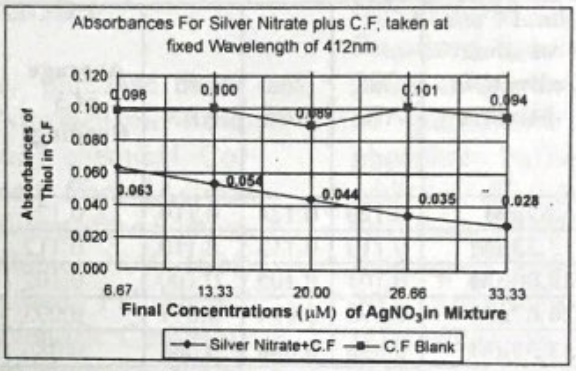

Figure 2- ***Curves For Control Thiol Level of Cytosolic Fraction & AgNo, effected Thiol Level of Cytosolic Fraction***

Table 3- Calculation for Concentration of GSH after reaction with Silver Nitrate by Ellman's Method

S/No.	Real Absorbance(ABS)	Concentration of GSH (μM) Remained in Cytosolic Fraction.
1	0.063	7.057
2	0.054	6.320
3	0.044	5.500
4	0.035	4.762
5	0.028	4.189

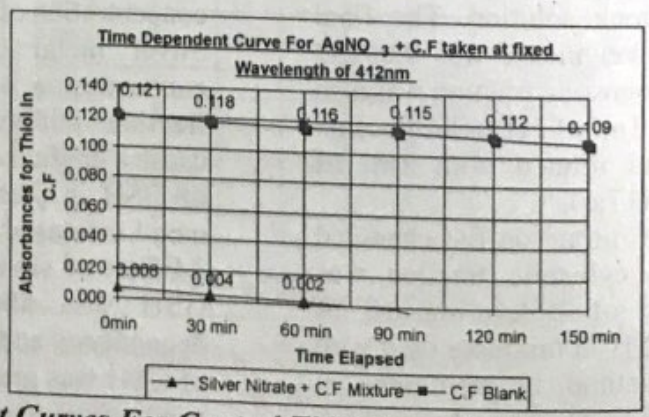

Figure 3- ***Time Dependent Curves For Control Thiol Level Of Cytosolic Fraction & AgNo3 affected Thiol Level of Cytosolic Fraction***

Table 4-Calculation for Concentration of GSH after reaction with Silver Nitrate by Ellman's Method

S/No.	Real Absorbance(ABS)	Concentration of GSH (μM) Remained in Cytosolic Fraction.
1	**0.008**	2.549
2	**0.004**	2.221
3	**0.002**	2.057
4	**0.000**	1.811
5	**0.000**	0.582
6	**0.000**	0.664

STATISTICALANALYSIS

Statistical Analysis for Effect of Silver on Glutathione (GSH) level in Cytosolic Fraction

Statistical approach for the effect of silver on the chemical status of GSH was also conducted for the concentration and time dependent effects.The paired comparison T-test (Table 5) of concentration dependent effect of silver and GSH blank gave the decision that there is an effect of silver on the chemical status of GSH in cytosolic fraction with increase in concentration of silver, as compared to GSH blank solution treatment. Similarly the Paired comparison T-test (Table 6) of time dependent effect of silver and GSH blank gave the decision that there is an effect of silver on GSH level in cytosolic fraction as the passage of time is increased with a specific concentration of silver as compared to GSH blank solution treatment.

Table 5- Paired comparison t-test for concentration dependent effect of AgNo3

	Affect of concentrations Of Silver on cytosolic Fraction of Glutathione	GSH(Blank)
Mean	0.044	0.096
Variance	0.00019	0.000024
Observations	5	5
Pearson Correlation	0.228	
Hypothesized Mean Difference	0	
df	4	
t Stat	-8.342	
P(T<=t) one-tail	0.00056	
t Critical one-tail	2.131	
P(T<=t) two-tail	0.0011	
t Critical two-tail	2.776	

Table 6- Paired comparison t-test for time dependent effect of silver nitrate

	Affect of concentrations Of silver on cytosolic Fraction of Glutathione with time	GSH(Blank)
Mean	-0.003	0.115
Variance	0.00010	1.816E-05
Observations	6	6
Pearson Correlation	0.941	
Hypothesized Mean Difference	0	
df	5	
t Stat	-46.132	
P(T<=t) one-tail	4.51E-08	
t Critical one-tail	2.0150	
P(T<=t) two-tail	9.03E-08	
t Critical two-tail	2.570	

DISCUSSION

There is increasing interest in GSH due to its varied physiological and pharmacological properties including detoxification through participation in the redox system, activation of SH-Enzymes, Co-enzymatic action and conjugation.Silver has been found to play a role in apoptosis (gene-directed cell death), a critical cellular regulatory process 'with implications for growth and development, as well as a number of chronic diseases. Cells in the salivary gland, prostate, immune system and intestine can secrete Silver

Thus it was of interest to study the interaction of this metal in vitro to establish further scientific data. This Scientific data about the interaction and the effect of Silver on the chemical modulation of GSH will enable us to understand further the role of Silver and GSH and strengthen our knowledge about their therapeutic uses in many diseases.

The study conducted showed that the concentration of GSH present in cytosolic fraction was shown to be low as compared to the level of GSH present in cytosolic fraction. The effect was same as viewed in the case of performance of effect of metal on GSH in aqueous medium and in cytosolic fraction.

Different concentrations of silver caused a decrease of concentration of glutathione and play important role in the conversion of GSH to either GSAg or GS-Ag-of reduced form of glutathione (SG) in cytosolic fraction. In the same manner the effect of silver was also time dependent on the chemical status of GSH and the concentration of reduced GSH present in cytosolic fraction was decreased with the passage of time. The following sequences of reactions (Equation 1) are suggested to be happened in the experiment.

Equation 1

GSH + Silver (Ag) ---> $(Ag)_4$-SG

The results also suggested that there was a possibility of formation of intermediate or conjugate between silver and GSH. However it was not possible to estimate or determine those conjugates under those conditions .Since both GSH and silver, is biological active compounds. It was of interest to study the possible interaction of this metal in vitro as a model of in vivo interaction.

CONCLUSION

The tripeptide thiole glutatine (GSH) has facile electron-donating capacity, linked to it sulfhydryl (SH) group. Glutathione is important water - phase antioxidant and essential cofactor for antioxidant enzyme. It provides protection also for the mitochondria against endogenous radicals. Its high electron donating capacity combined with its high molecular concentration endows (GSH) with great reducing power, which is used to regulate a complex thiole-exchang system. Different concentration of Silver metal caused a gradual decreased in the concentration of GSH in cytosolic fraction. Effect of silver on the chemical status of glutathione was also studied for the time dependency and noted that the concentration of glutathione gradually decreased as the time passes from (0 minute interval of time to 150minutes) in cytosolic fraction.

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