REPORT

Study of the effects of $BaCl_2$ on the level of glutathione in plasma and cytosolic fraction in whole blood

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Abstract: Barium has important role in the field of medical sciences, but it has been found in various studies that barium can cause numerous toxic effects. Studies have proven the strong affinity of the metalloelements for the sulfhydryl group (SH), present in reduced glutathione (GSH) and other biological molecules. In this context, the study about the possible interaction of $BaCl_2$ with glutathione in whole blood components was of interest, as an indication about the extent of barium toxicity and the role of glutathione in the conjugation and detoxification of the metalloelement barium. The concentration dependent and time dependent effect of $BaCl_2$ on the level of GSH in plasma and Cytosolic Fraction in whole blood was investigated, following Ellman's method. It was found that $BaCl_2$ causes a decrease in the GSH level, which is more pronounced with increasing concentration of $BaCl_2$ and with time incubation as well. The observed effect GSH concentration may be presumably due to production of oxidized glutathione (GSSG) or then due to Barium-Glutathione (GS-Ba-SG) conjugate formation.

Keywords: Glutathione (GSH), 5, 5-Dithiobis-(2-Nitrobenzoic acid) (DTNB), barium chloride ($BaCl_2$), absorbance (ABS)

INTRODUCTION

Barium, a divalent alkaline earth metal, can not occur freely in nature (Weast *et al*., 1987). It occurs in combination forms like nitrate, sulfate, chlorate, peroxide and chloride (Weast *et al*., 1987). In different studies, it was reported that human expose to barium through drinking water (Fonds *et al*., 1987; Lanciotti, 1992), through air (Apostoli *et al*., 1998; Dare *et al*., 1984), through food (Mertz, 1986; Murphy *et al*., 1971) and through diagnostics (IPCS, 1990).The total daily intake of barium from food, drinking water and air is 1.33mg barium/day (Schroeder *et al*., 1972). Barium is used as medical contrast medium in computed tomography (Buckwalter and Kenneth, 2000) and fluoroscopic analysis (Shroy and Robert, 1995) of human body, to improve the visibility of scans and x-ray images (Thomsan and Varma, 2010). Thus barium is employed in the diagnosis of different diseases, including crohn's disease (Baumgart and Sandborn, 2007), achalasia (Spiess and Kahrilas, 1998), gastro esophageal reflux disease (Numans *et al*., 2004), esophageal cancer (Enzinger and Mayer, 2003) and Schatzki ring (Pezzullo *et al*., 2003).Barium compounds like barium chloride and barium sulfate get absorbed from the gastrointestinal tract (Lisk *et al*., 1988; McCauley and Washington, 1983) and from the respiratory tract (Cuddihy and Griffith, 1972).

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Barium act as competitive antagonist of potassium channel (Roza and Berman, 1971) and have been found to cause different toxic effects in different animal and human studies. These toxic effects include focal thickening of the ineralveolar septa, peribronchial sclerosis (Tarasenko *et al*., 1977), arrhythmias (Deng *et al.,* 1991), gastric pain and gastrointestinal hemorrhage (Diengott *et al*., 1964), decreased thrombocyte count (Tarasenko *et al*., 1977), muscle paralysis of neck and extremities (Shankle and Keane, 1985), glomerular lesions (McCauley *et al.,* 1985), decreased serum potassium level (Zschiesche *et al*., 1977), increased blood phosphorous and glucose level, decreased urinary calcium level (Tarasenko *et al*., 1977), absence of deep tendon reflexes (Shankle and Keane, 1988), tingling and numbness around the mouth (Lewi and Bar-Khayim, 1964) and brain edema (McNally, 1925), decrease in sperm count and morphological alterations in ovaries (Tarasenko *et al*., 1977) and retinal dystrophy (McCauley *et al*., 1985). In a case, cervical dysplasia was induced in a female due to application of barium chloride (Ayre, 1966). Glutathione is a tripeptide, containing an unusual peptide linkage of the amine group of amino acid cysteine to the carboxyl group of amino acid glutamic acid side chain (Pompella *et al*., 2003). Glutathione exists in glutathione reduced (GSH) and glutathione oxidized (GSSG) forms in the body, among which; the GSH is more in concentration than GSSG (Kaplowitz *et al*.,

1985). In mammalian cells, GSH is the dominant non-protein thiol (Lu, 1999). The glutathione level ranges from 0.1 to 10 millimolar (mM) in different human tissues (Verjee and Behal, 1976). Glutathione is synthesized in most of the body cells and also transported from the liver to various other organs of the body (Griffith and Meister, 1979; Meister, 1983). The most important function of GSH is detoxification of xenobiotics and drugs by GSH conjugation reaction (Meister, 1988).

GSH binds to heavy metals, like zinc, mercury, cadmium and copper, Lithium, Aluminum, and Silver and transport them to the liver, where they are further detoxified and excreted (Agrawal *et al.,* 2007, Haroon *et al*., 2010, 2011a, 2011b). The oxidation state of barium is 2^+ (Robert *et al*., 2007) and GSH has electron donating property (Kidd, 1997). Thus this study was performed to reveal the possible interaction of barium metalloelement with the glutathione in plasma and Cytosolic fraction in whole blood. Barium chloride was selected for the study because of its solubility in water ($BaCl_2$, 2010).

MATERIALS AND METHODS

Materials

L.Glutathione (GSH) (Fluka), 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) (sigma), Hydrochloric acid 35% (HCl) (Merck), Sodium hydroxide (Na0H) (Fluka AG), Barium chloride ($BaCl_2$) (Peking chemical works, China), Potassium dihydrogen phosphate (Merck), Disodium edetate (Riedle Dehean Ag sleeze Hannover), Chloroform (Merck), Ethanol (Merck), Sodium chloride (Merck) and Distilled water (double refined).

Different Glass wares were required, including flasks of different volumes, graduated cylinders, pipettes, Test tubes, Beakers, funnels, etc.

All the reagents were used as purchased without any further purification. The glass wares were properly washed with detergent powder, distilled water, and organic solvents and dried in oven for a time of 2 hours at a temperature of 110^{o}C.

Different Instruments were required, including, pH Meter (Model NOV-210) Nova Scientific Company Ltd., Korea, Oven (Memmert Model U-30), 854 Schwabach (Germany), UV-1601 (Schimadzu), automatic double beam Spectrophotometers (Japan), Graduated Micropipette (Scorex Swiss Finland), Magnetic stirrer, hot plate 400 (England), Sartorius Balance, Eppendolf's tubes (Plastic 101), Siliconazed Glass test tubes, Centrifuge (H-200, Kokusan Ensink Company, Japan), Disposable rubber gloves and sterile pyrogen free disposable syringes (B.D).

Methods

Preparation of required solutions

100ml of 1mM GSH solution was prepared by dissolving 30.74mg of GSH (molecular weight 307.4) in 0.1N HCl solution. 42.4ml of 0.2M Na0H solution was mixed with 50ml of 0.2M Monobasic Potassium Phosphate solution and diluted up to 200ml with distilled water to prepare 0.2M phosphate buffer pH 7.6 stock solution. 0.9% NaCl solution (Normal saline) was prepared by dissolving 0.90gm (900mg) of the pharmaceutical grade sodium chloride (NaCl) in distilled water and diluting up to 100ml with distilled water. 100 ml of 1mM 5,5-Dithiobis-(2-Nitrobenzoic acid) (DTNB/Ellman's reagent) was prepared by dissolving 39.6mg of DTNB (molecular weight 396.35); in 0.2M buffer (pH 7.6). 1mM isotonic solution of Barium chloride (molecular weight 244.28) was prepared by dissolving 0.24428gram (244.28 milligram) of $BaCl_2$ in Normal saline and was diluted up to 1000ml with 0.9% NaCl solution. To obtain 0.5M disodium edetate (EDTA-2Na, molecular weight 372.2) 1.861gm of it was dissolved in 10ml of distilled water). Then 1ml of it was diluted up to 100 ml with distilled water to obtain 5mM disodium edetate solution. For to prepare 80ml of 3:5 Chloroform: Ethanol mixture, 30ml of the Chloroform was mixed with 50ml of Ethanol.

Isolation of plasma

12ml of blood was collected from the vein of a healthy volunteer and was mixed with 0.5ml (500µl) of 0.5M sodium edetate to prevent its clotting. 1ml of each concentration of barium chloride (0.2mM-1mM) was mixed separately with 1ml of blood. Thus in this way we prepared 5 test tubes. Final concentrations of $BaCl_2$ were 100µM-500µM in these test tubes. Each of these samples was centrifuged for 5 minutes at 10,000 rpm. 0.8ml of the supernatant (plasma) was collected carefully from each of these mixtures after centrifugation and kept on ice in a sample tube till use. The remaining packed cells volume was used further for the isolation of Cytosolic Fraction. For plasma control, 1ml of blood was mixed with 1ml of normal saline, centrifuged and plasma was collected.

Isolation of cytosolic fraction of blood

Each of the packed cell volume was washed two times with normal saline and was lysed with an equal volume of distilled water for 1 hour at 4^{o}C. Then 0.8ml of cold Chloroform: Ethanol mixture was added to each of these lysed cells volume, so as to precipitate hemoglobin. Then 0.3ml of distilled water was added to each of them and the resulting mixtures were centrifuged for 5 minutes at 10,000 rpm. After centrifugation, the supernatant pale yellow (Cytosolic Fraction) was collected carefully and stored on ice in a sample tube, till use. The packed cell volume from previously centrifuged sample for isolation of plasma control was further processed for collection of Cytosolic Fraction control. The Cytosolic Fraction was collected by the mentioned procedure.

Determination of biochemical inorganic parameters

1. Plasma glutathione (extracellular)
2. Cytosolic Fraction glutathione (Intracellular)

All the estimations of glutathione were carried out following the modified standard Ellman's method (Ellmans, 1959) as follows. 0.2ml of each of the sample (Plasma or Cytosolic Fraction of blood) was mixed with 2.3ml buffer and 0.5ml of DTNB. Each of these mixtures was transferred to spectrophotometric cell one by one. The reference cell was contained with buffer. All the measurements were taken at 412nm, by using a U.V-visible spectrophotometer model 1601 (Schimadzu). A DTNB blank, containing 0.5ml of 1mM DTNB and 2.5ml of buffer was also run against a reference cell containing buffer, at 412nm.The concentration of glutathione in each sample was using the standard curve.

Ethical consideration

In this work, 3 male volunteers were selected (ageing 25-35 years). Prior to the tests, the volunteers were examined by a physician for any serious disease. Before the study, every volunteer was provided with a volunteer protocol. This protocol stating the terms and conditions of the testing were signed by every volunteer individually. All the process was done at 25°C and 40% relative humidity conditions. The study approved by institutional committee in accordance NIH guidelines. Reference No. GU/0311/DIK

Standard Curve

Following the Ellman's method, we prepared the standard curve by using 100µM-500µM of GSH.

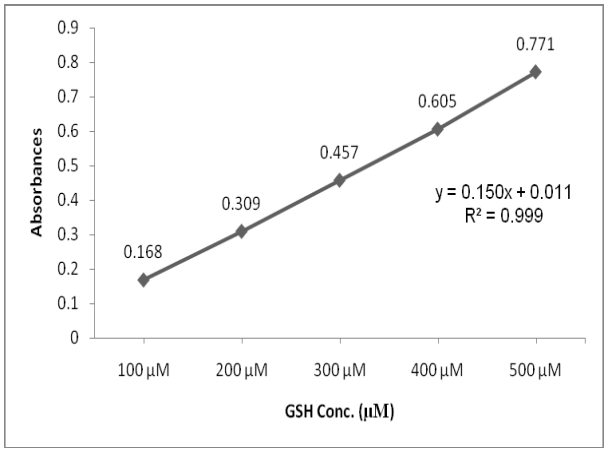


Fig. 1: Standard calliberation curve for GSH at 412nm

Effect of Barium chloride on the extracellular plasma GSH content

Each of the plasma sample (collected by the isolation process as described above), containing increasing concentrations of $BaCl_2$ in the range of 100µM-500µM was investigated for GSH content through U.V-visible spectrophotometer following the already described Ellman's method (Ellmans, 1959).

We observed that a slight decrease in the GSH concentration was caused by the $BaCl_2$, which was gradually increasing with increasing concentration of the $BaCl_2$, as shown in fig. 2.

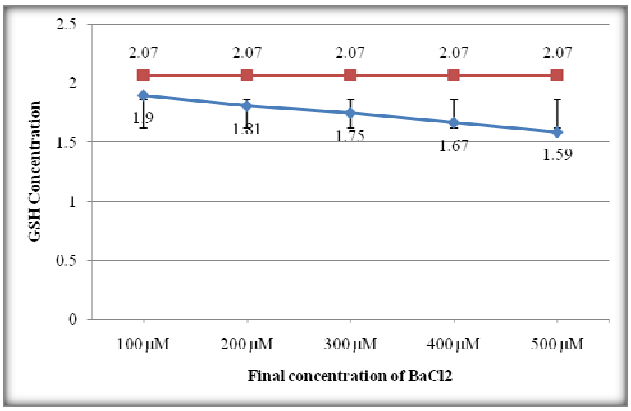


Fig. 2: Effect of $BaCl_2$ concentration on extra-cellular plasma GSH Content

■Control (1ml 0.9% NaCl/1ml of blood)

♦ $BaCl_2$ (100-500 µM)

Results are the mean ± SEM of 3 experiments of plasma GSH

A significant change was observed in the plasma GSH content from the control at $P<0.5$.

For time-dependent study, the GSH content was measured (by the described procedure) in two different samples, containing 100µM and 500µM of $BaCl_2$ at time intervals 0-150 minutes. Again a decrease was found in plasma GSH content with time as shown in fig. 3.

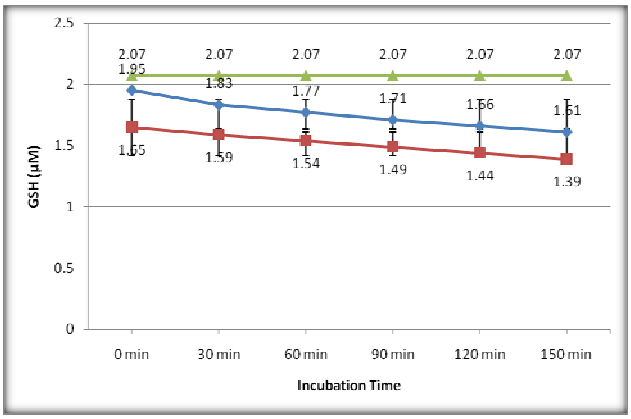


Fig. 3: Effect of $BaCl_2$ Concentration on the extra-cellular plasma

GSH content with time incubation period (0-150 minutes)

▲Control (1ml 0.9% NaCl/1ml of blood)

♦ $BaCl_2$ (100µM)

■ $BaCl_2$ (500µM)

Results are the mean ± SEM of 3 experiments of plasma GSH

Effect of Barium chloride on the intracellular Cytosolic Fraction GSH content

Each of the collected Cytosolic Fraction sample, containing different concentrations of $BaCl_2$ (100µM-500µM) was investigated for GSH content through U.V-visible spectrophotometer following the Ellman's method (Ellmans, 1959).

We found that $BaCl_2$ has caused a slight decrease in the GSH concentration. This decrease was gradually increasing with increasing concentration of the $BaCl_2$ as shown in fig. 4.

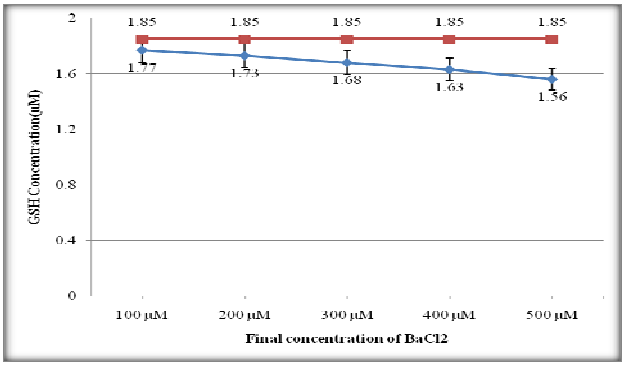


Fig. 4: Effect of $BaCl_2$ Intra-cellular cytosolic fraction GSH content.
■Control (1ml 0.9% NaCl/1ml of blood), ♦ $BaCl_2$ (100-500 µM)
Results are the mean ± SEM of 3 experiments of Cytosolic Fraction GSH

A significant change was observed in the Cytosolic Fraction GSH content from the control at $P<0.5$.

For to know the time-dependent effect of barium chloride on the GSH content in Cytosolic Fraction, the content of GSH was measured in two different samples, containing 100µM and 500µM of $BaCl_2$ at time intervals 0-150 minutes. Again a decrease was found in Cytosolic Fraction GSH content with time as shown in fig. 5.

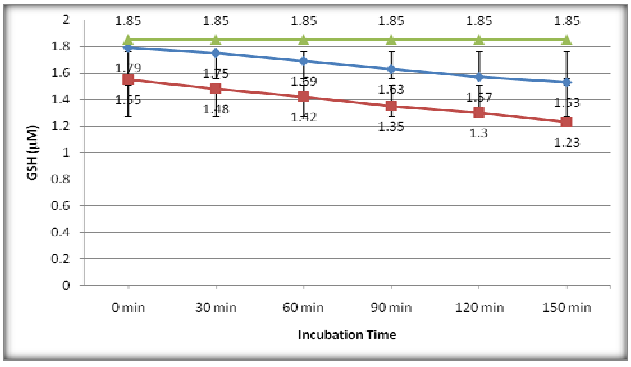


Fig. 5: Effect of $BaCl_2$on intracellular cytosolic fraction.
GSH content with time incubation period (0-150 minutes)
▲Control (1ml 0.9% NaCl/1ml of blood), ♦ $BaCl_2$ (100µM)
■ $BaCl_2$ (500µM)
Results are the mean ± SEM of 3 experiments of Cytosolic Fraction GSH

STATISTICAL ANALYSIS

Statistical analysis for the effect of $BaCl_2$ on the chemical status of (GSH) was also conducted for the plasma and cytosolic fraction GSH level and found that using the 95% confidence interval level.

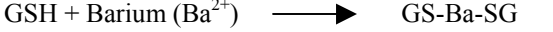
Results are presented as a mean ± SEM. Statistical significance and difference from control (GSH in plasma) and test values (GSH in plasma + barium chloride) and GSH in cytosolic fraction) and test values (GSH in cytosolic fraction + barium chloride) (evaluated by Student's paired t- test gave the decision that there is an effect of $BaCl_2$ on GSH level in plasma and cytosolic fraction of whole blood as compared to blank plasma and cytosolic fraction. 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 level of significance

DISCUSSION

The observations were recorded at different concentration and 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. This study shows metalloelement (Barium) has concentration and time wise decreased the level of GSH in Plasma and cytosolic fraction. Glutathione has a reducing capacity for exogenous compounds like Barium and converts itself to oxidized state which is a disulphide (GSSG). The purpose of this research work was to know about the possible interaction between glutathione and barium metal.

Barium is used as a medical contrast medium in the diagnosis of different diseases in fluoroscopy (Shroy and Robert, 1995) and computed tomography (Buckwalter *et al*., 2010). Human body can also be exposed to barium through drinking water, air and food (Schroeder *et al*., 1972). Findings from different studies have shown that barium can cause various toxic effects (Tarasenko *et al*., 1977; Shankle and Keane, 1988). Our study indicates decrease GSH level in plasma (figure 2) and Cytosolic fraction (figure 3) due to Barium. The decrease in GSH level in cytosolic fraction due to $BaCl_2$ 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).The importance of interaction of metalloelements including $BaCl_2$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

One of the important functions of glutathione is its role in the metabolism of metals (Agrawal *et al.*, 2007). We also observed that this effect of $BaCl_2$ was gradually increasing with increasing concentrations of $BaCl_2$. We also performed time-dependent observations and again a gradual decrease was observed in the concentration of GSH with time. Considering the reducing power of GSH (Kidd, 1997) and the 2^+ oxidation state of barium (Robert Kresse *et al.,* 2007), we can presume a possible conjugation reaction between reduced GSH and barium metal. A hypothetical sequence of this reaction can be suggested as given below.



Although the exact mechanism of this conjugation reaction can not be distinguished from this piece of research work, however this study can be useful further to explore the role of GSH in the metabolism and excretion of the important diagnostic agent barium. This research study also gives an idea about the protective role of GSH against barium toxicity.

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