Depletion of GSH in human blood plasma and cytosolic fraction during cadmium toxicity is temperature and pH dependent

Hashmat Ullah[1], Muhammad Farid Khan[1], Syed Umer Jan[2]* and Farwa Hashmat[3]

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy Gomal University, Dera Ismail Khan, Pakistan
[2]Faculty of Pharmacy, University of Balochistan, Quetta, Pakistan
[3]Department of Chemistry, Gomal University, Dera Ismail Khan, Pakistan

Abstract: Toxicities of heavy metals is a burning issue and a topic of interest among the toxicologists throughout the world. Metals are always in use of man since long but in recent years the use of cadmium has increased in the form of various cadmium compounds such as cadmium compounds as stabilizers in plastic pipe industries and in the preparations of different alloys etc. Cadmium is even used in phosphate fertilizers and thus comes directly or indirectly in contact with human eatables like crops, vegetables and fruits. Once it is absorbed it affects almost all the organs and systems of human body especially blood components and kidneys. Always the chemical reactions of different chemicals are dependent on some influential factors, among these factors the effect of pH and temperature of the media in which these chemicals interact with each other are very much important. Keeping in view this fact we have evaluated the effect of cadmium nitrate tetra hydrate on GSH of human plasma and cytosolic fraction. Estimation of thiol was done by Ellman's modified method and was found that the interaction of cadmium nitrate tetra hydrate and GSH of these blood components was more at a pH and temperature, which were near to physiological pH and temperature of human body. This fact was proved as the estimated thiol concentration left after the interaction of cadmium nitrate tetra hydrate and thiol of these blood components was minimum at pH and temperature near to human blood pH and temperature. We concluded that the possible reason for depletion of GSH of these blood components was conversion of GSH into $Cd(SG)_2$and/or GSSG formation.

Keywords: Blood plasma, temperature, estimation, reduced glutathione, modified, physiological pH.

INTRODUCTION

All environmental agents like non-genotoxic carcinogens can directly generate or indirectly induce reactive oxygen species in cells (Kidd, 1997). The oxidative stress and damage has been observed following exposure to various xenobiotics including chlorinated compounds, metal ions, radiation and barbiturates (Stohs and Blichi, 1993). Many studies have focused on metal-induced toxicity and carcinogenicity, emphasising their role in the generation of reactive oxygen and nitrogen species in biological systems (Valko *et al*., 2005; Leonard *et al*., 1994; Chung., 2005; Halliwell and Gutteridge, 1990; Khan H *et al.,* 2010, 2011a, 2011b and 2012; Muktiar *et al., 2012 and* 2013; Shah *et al.,* 2013 & 2013a; Khan J *et al.,* 2012 and Naseem *et al*., 2015). Metal-mediated formation of free radicals may cause various modifications to DNA bases, enhanced lipid per oxidation, and changes in calcium and sulphydryl homeostasis (Shaham *et al*., 1996). It is known that metal-induced generation of oxygen radicals results in the attack of not only DNA in the cell nucleus, but also other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation and (Moriarty *et al*., 2004). ROS and metal ions primarily inhibit phosphor-serine/threonine-, phosphotyrosine- and phospholipid-phosphatases, most probably by interacting with sulphydryl groups on their cysteine residues, which are oxidized to form either intramolecular or intermolecular disulphide bonds (Ercal *et al*., 2001). Generally, the antioxidant capacity of thiol compounds is due to the sulphur atom, which can easily accommodate the loss of a single electron (Wasowicz *et al*., 2001). In addition the lifetime of sulphur radical species thus generated, i.e. a thiyl radical (GS•), may be significantly longer than many other radicals generated during the stress (Sen, 1998). Oxidised glutathione GSSG is accumulated inside the cells and the ratio of GSH/GSSG is a good measure of oxidative stress of an organism (Hwang *et al*., 1992). Cadmium is well thought-out as one of the most toxic substances in the environment owing to its ample range of organ toxicity and long elimination half-life of 10-30 years (Jarup *et al*., 1998). Cadmium has long biological half-life in human and it accumulates mainly in liver and kidneys where it damages both these vital organs (Firberg, 1984; Lash *et al*., 1999). The uptake of cadmium from the soil though produce results in elevated concentrations in vegetables, fruits, and grains, with the highest levels in leafy greens and potatoes (Nordberg, 1984). High levels are also found in shellfish (up to 30mg/kg) and organ meats (Ercal *et al*., 2001). Keeping in view the toxicity and chemical affinity of cadmium towards thiol especially to glutathione, it was very interesting to investigate the effect cadmium nitrate tetra hydrate on reduced

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

glutathione of human blood plasma and cytosolic fraction in various selected pH buffers and at different temperatures.

MATERIALS AND METHODS

Material

Following were the chemicals, reagents and instruments used in this research work.

Chemicals and reagents

Ethanol (Merck)**,** Disodium edetate (Riedel Dehean AG sleeze Hannover), 5,5-dithiobis-(2-nitrobenzoic acid, (Sigma), Glutathione (Fluka), NaOH (Fluka AG), KH_2PO_4 (Merck), NaCl (Merck)**,**Water for injection (Elixir Laboratories), Hydrochloric acid 35% (Kolchlight), Distilled water (double refined), Chloroform (Merck), Cadmium nitrate tetra hydrate (Aldrech).

Instruments

Nov-210 pH meter (Nova scientific Company Ltd korea), Analytical balance Model AX 200 (schimadzu, Japan), Centrifuge H-200 (Kokusan Ogawa seiki Co., Ltd Japan), Magnetic stirrer, Siliconazed glass test tubes, Disposable rubbergloves (otsuka, Japan) automatic double beam Schimadzu, UV-1601 Spectrophotometer (Japan), Memmert oven (Model U 30854 Schwa Bach, Germany), Eppendol's tubes plastic 100µl, 200µl, 500µl, 1000µl Micro pipette (Scorex Swiss Finland), Sterile pyrogen free disposable syringes.

Methods

The methods used in this research work were:-

i. Preparation of different stock solutions

The stock solutions prepared for this research work were; 0.9% sodium chloride solution, 0.2M sodium hydrooxide, 0.2M KH_2PO_4, 0.1N HCl solution, 1mM Glutathione (Molecular weight 307.4) solution, 1mMDTNB (Molecular weight 396.35), 2mM cadmium nitrate tetra hydrate isotonic solution was prepared by dissolving 47.2mg in 100ml of normal saline. 30ml of chloroform was mixed with 50ml of ethanol to prepare 3:5chloroform: ethanol mixture (1:1), EDTA, 0.5mM(Na-ethylene diamine tetra acetic acid, molecular weight 372.2). From 2mM stock solution of cadmium nitrate tetra hydrate, 6 different dilutions were prepared (0.0001,0.001,0.01. 0.1,1.0 and 2.0mM).

ii. Isolation and separation of plasma and cytosolic fraction of human blood

a. Plasma fraction

Fresh venous blood of healthy human volunteer's exactly 8ml was mixed with 500µl of 0.5M disodium edetate to evade any clotting of the blood in process. Six test tubes were taken and in each of the test tubes 1000µl of blood was mixed with 1000µl of different cadmium nitrate tetra hydrate from 0.0001mM to 2.0mM. In this way we obtained six mixtures (Blood plus CNT) with different concentration of cadmium nitrate tetrahydrate (CNT), these mixtures were well shaken and incubated for 10minuts and after 10 minutes all of them were centrifuged at 10,000 rpm for 5 minutes. The supernatant layer in each mixture was plasma. From the supernatant layer (plasma) of each mixture 800µl was cautiously taken by using 1000µl pipette and shifted to separate sample test tubes and the remaining packed cells fractions were further processed for the collection of cytosolic fractions.

b. Cytosolic fraction

The packed cells were processed for cytosolic fractions which were thoroughly washed three times with 1200µl of 0.9% normal saline solution.1200µl of distil water was also added slowly drop wise in order to lyse red blood cells. Cold chloroform and ethanol mixture (3:5 v/v), 0.6ml at $4C^0$, was added to precipitate hemoglobin. The obtained fraction from each sample was centrifuged and the pale yellow supernatant cytosolic fraction was separated from each mixtures. These cytosolic fractions were transferred to sample test tubes for use in this experiment.

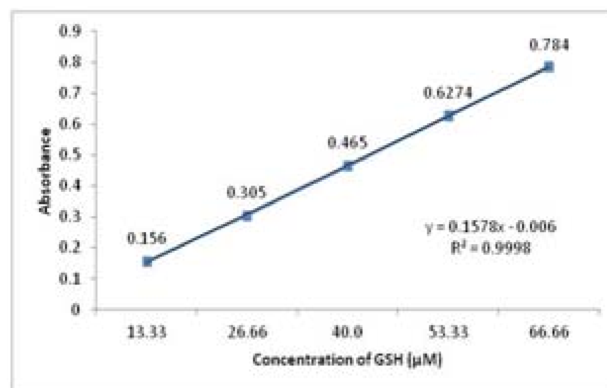

Fig. 1: Standard Curve for Glutathione (GSH)

iii. Experimental procedure

For reaction mixture, reaction buffers having four different pH ranges i.e. 6.5, 7.0, 7.5 and 8.0 were used to investigate the effect of cadmium nitrate tetra hydrate blood plasma and cytosolic fraction. The reaction mixtures for investigation of pH dependent effect were incubated for 10 minutes and sample mixture for 5 minutes and after the passage of incubation time of sample mixture, the absorbance of each sample was recorded at fixed wave length λmax; 412nm against buffer of respective pH as a reference under UV-visible spectrophotometer. The relative absorbance of DTNB blank solution was also measured at λmax: 412nm and it was subtracted from the absorbance of mixture of cadmium nitrate tetra hydrate with GSH in presence of DTNB to get the real absorbance of GSH left after the interaction of different dilutions of cadmium nitrate tetra hydrate with plasma/ cytosolic fraction GSH.

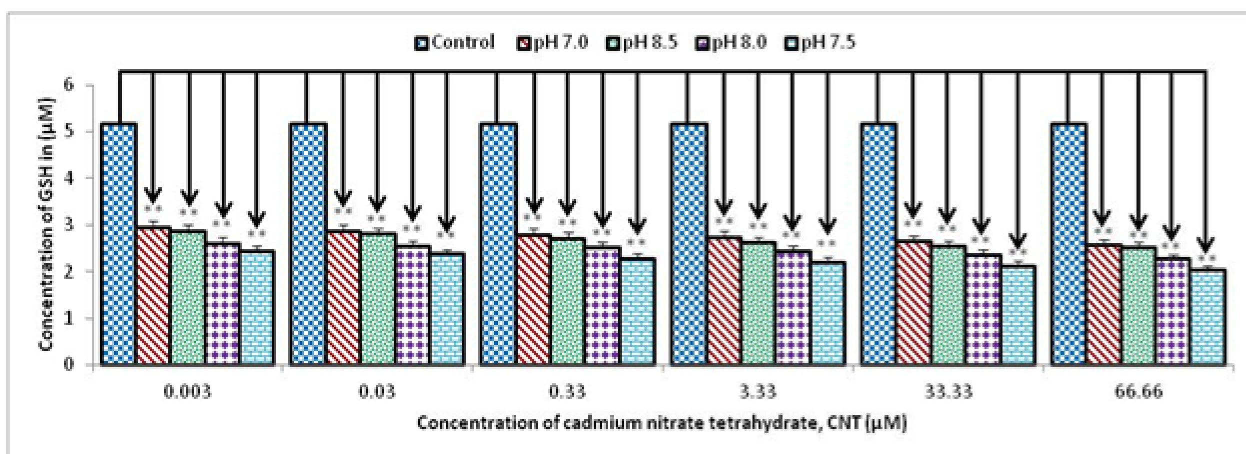

Fig. 2: pH dependent effect of cadmium nitrate tetra hydrate, CNT (0.0001-2.0mM) on plasma GSH contents. Left to right first long bars show plasma control (Mean ±SD, n=3)

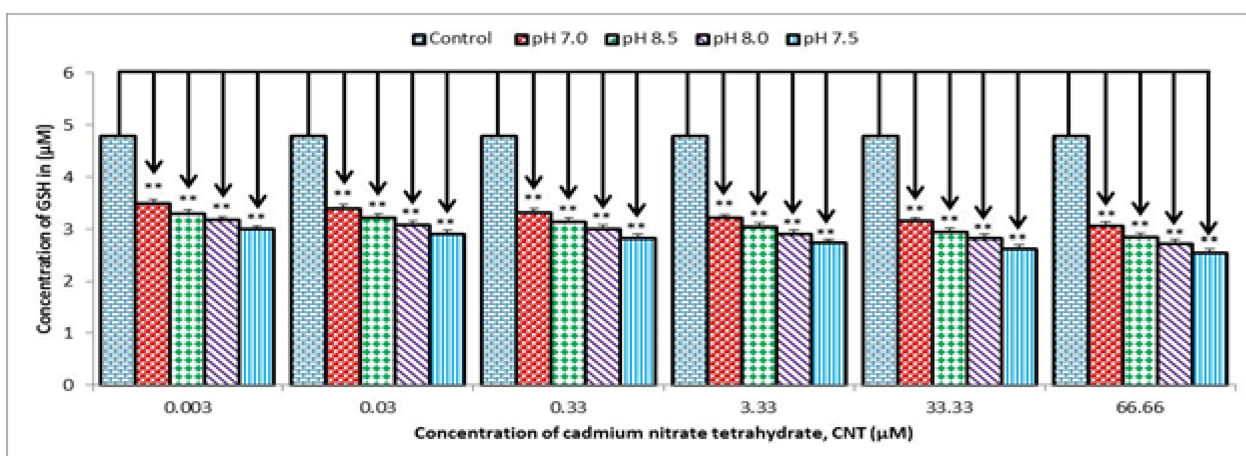

Fig. 3: pH dependent effect of cadmium nitrate tetra hydrate, CNT (0.0001-2.0mM) on cytosolic fraction GSH contents. Left to right first long bars show cytosolic fraction control (Mean ±SD, n=3)

a. Plasma/cytosolic control

Control sample was prepared for pH dependent effect in buffer of respective pH by mixing 1ml of isolated blood plasma/ cytosolic fraction with 1ml of reaction buffer of respective pH (equal volume) 200µl of this mixture was mixed with 2300µl of buffer of respective pH following by the addition of 500µl of DTNB. The absorbance of control sample was also recorded at 412nm against reaction buffer as reference in UV-visible spectrophotometer.

iv. ***Standard curve for glutathione***

Standard curve for GSH was obtained by Ellman's method. After preparing 1mM glutathione (GSH) solution, its different dilutions from 1mM GSH stock were made like 0.2mM, 0.4mM, 0.6mM and 0.8mM and from each GSH dilution 200µl was mixed with 2300 µl of phosphate buffer pH 7.6 following by slow mixing of 500µl of 1mM DTNB (5,5-dithiobis-2-nitrobenzoic acid). These were the sample mixtures, which were well shaken and incubated for 5 minutes. The final concentration of GSH in these mixtures was 13.33µM, 26.66µM, 40µM, 53.33µM and 66.66µM respectively. After incubation time of 5 minutes, absorbance was taken at fixed wave length λ max: 412 nm and as a result following standard curve was obtained.

a. DTNB blank

500µl of 1.0mM DTNB in 2500µl of phosphate buffer pH 7.6 was mixed. Absorbance of DTNB blank was also taken at fixed wave length λ max: 412 nm.

b. Real absorbance

By subtracting absorbance of DTNB (5,5-dithiobis-2-nitrobenzoic acid) from absorbance of glutathione plus DTNB (5,5-dithiobis-2-nitrobenzoic acid), the resulting absorbance is called as real absorbance.

v. Estimation of GSH (Glutathione) concentration in blood component

The remaining concentration of reduced glutathione left after its interaction with different concentrations of

cadmium nitrate tetra hydrate was estimated by using already published Ellman's method (Ellman's., 1959).

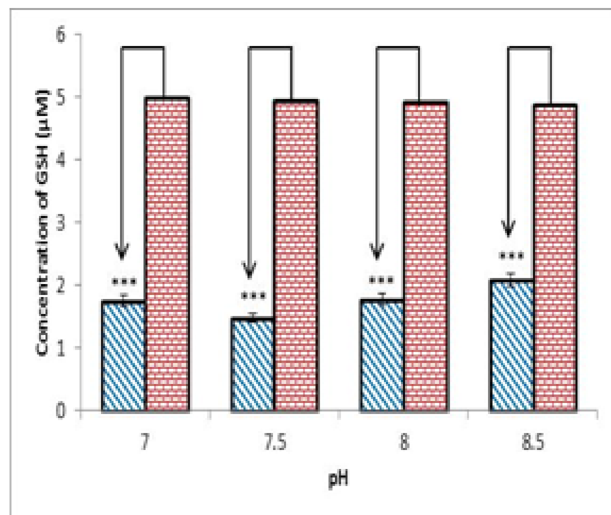

Fig. 4: pH-dependent effect of cadmium nitrate tetra hydrate (0.1mM) on cytosolic fraction GSH in 0.1 M, KH_2PO_4, buffers of different pH. Righr to left long bars show cytosolic fraction control (Mean ±SD, n=3).

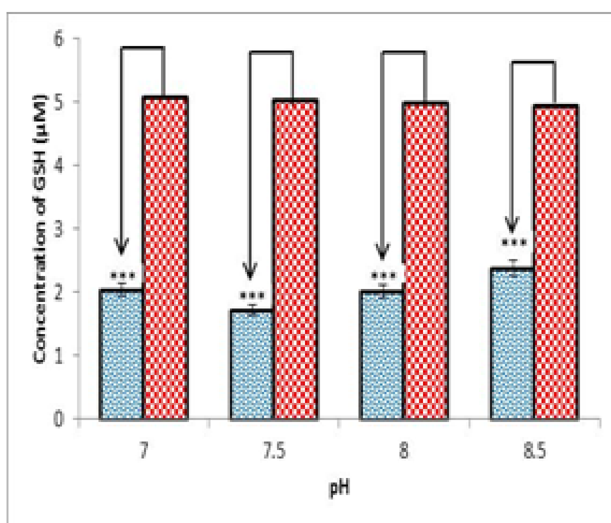

Fig. 5: pH-dependent effect of cadmium nitrate tetra hydrate (0.1mM) on plasma GSH in 0.1M, KH_2PO_4, buffers of different pH. Righr to left long bars show cytosolic fraction control (Mean ±SD,

a. Plasma / Cytosolic Glutathione determination

By mixing 200µl of each sample mixture containing plasma and cadmium nitrate tetra hydrate/cytosolic fraction and cadmium nitrate tetra hydrate with 2300 µl phosphate buffer pH 7.6 following by the slow mixing of 500µl of 1mM DTNB (5,5-dithiobis-2-nitrobenzoic acid) solution, the resulting sample mixtures were well shaken and incubated for 5 minutes then were shifted one by one to the cuvette of UV-Visible spectrophotometer and absorbance was recorded at fixed wave length λ max: 412 nm against reference cell contained buffer pH 7.6 and the recorded absorbance was converted into GSH concentration by using standard curve (Ellman's method, 1959).

RESULTS

i. pH dependent effect of cadmium nitrate tetrahydrate on GSH of blood plasma and cytosolic fraction

Effect of various concentration of cadmium nitrate tetra hydrate on plasma/cytosolic fraction level of GSH was investigated in phosphate buffers of four different pH, which were 7.0,7.5, 8.0 and 8.5 and it was very interesting to note that the interaction between various concentrations (0.0001-2.0mM) of cadmium nitrate tetra hydrate and GSH level of isolated plasma/cytosolic fraction was fast in buffer of pH 7.5.In case of isolated plasma GSH level was decreased statistically significantly ($p<0.001$) in buffer pH 7.5 which was 2.994 µM (62.60%), 2.911µM (60.86%), 2.828µM (59.13%), 2.739µM (57.26%), 2.637µM (55.13%) and 2.548µM (53.27%) by different used concentrations of cadmium nitrate tetra hydrate respectively with respect to control (pH 7.5) as compare to plasma GSH level drop in other used buffers of pH 7.0, 8.0 and 8.5 with respect to their respective control (table 1). The drop in plasma GSH level in these buffers of pH 7.0, 8.0 and 8.5 by various concentrations of cadmium nitrate tetra hydrate is shown in detail in fig. 2. The drop in cytosolic fraction GSH level by various used concentrations (0.001-2.0mM) of cadmium nitrate tetra hydrate (CNT) was more in buffer pH 7.5 which was 47.21%, 45.74%, 44.09%, 42.41%, 40.94% and 39.08% while the drop in cytosolic fraction GSH level by these used concentrations of cadmium nitrate tetra hydrate in buffers with other pH (7.0,8.0,8.5) is shown in detail in fig. 3. Although concentration of GSH is high inside the RBCs as compare to plasma but still the drop in GSH level of cytosolic fraction is more as compare to drop in GSH level of plasma indicating that enough cadmium has crossed the semi permeable membrane of red blood cells and has caused this much depletion in GSH level of cytosolic fraction of blood.

ii. Temperature dependent effect of cadmium nitrate tetra hydrate on GSH of blood plasma and cytosolic fraction

Temperature has great impact on the chemical reactions and especially the human body temperature has critical role on the rate of chemical reactions taking place inside the human body. Due to this fact the interaction of cadmium nitrate tetra hydrate with plasma and cytosolic fraction GSH was investigated at three different temperatures, which were 25°C, 37°C and 45°C. It was found that the interaction between cadmium nitrate tetra hydrate and isolated blood plasma/cytosolic fraction was fast and depletion of GSH in both the compartments was greater at temperature 37°C which is human body

Table 1: pH dependent effect of all the used concentrations of cadmium nitrate tetra hydrate (CNT) in buffer pH 7.5 on the chemical status of isolated plasma and cytosolic fraction GSH. Results are the mean ± SE of 3 experiments.

Concentration Used of inorg/org Salts of cadmium	Final Conce: (in reaction mixture) of Inorganic/ Org Salt of cadmium	Final Conce: (in sample mixture) of inorg/org Salts of cadmium	Inorganic Salt of cadmium (CNT)	Plasma GSH Control	Remaining %age of GSH	Inorganic salt of cadmuim (CNT)	Cytosolic fraction GSH Control	Remaining %age of GSH
0.0001mM	0.00005mM	0.003µM	3.42	4.68	73.10	3.11	5.06	61.46
0.001mM	0.0005mM	0.033 µM	3.08	4.68	65.81	2.62	5.06	51.78
0.01mM	0.005mM	0.33 µM	2.78	4.68	59.40	2.31	5.06	45.65
0.1mM	0.05mM	3.33 µM	2.07	4.68	44.23	1.81	5.06	35.77
1.0mM	0.5mM	33.33 µM	1.55	4.68	33.12	1.48	5.06	29.25
2.0mM	1.0mM	66.66 µM	1.22	4.68	26.10	1.01	5.06	19.96

Table 2: Temperature dependent effect of all the used concentrations of cadmium nitrate tetra hydrate (CNT) at temperature 37°C on the chemical status of isolated plasma and cytosolic fraction GSH. Results are the mean ± SE of 3 experiments.

Concentration Used of inorg/org Salts of cadmium	Final Conce: (in reaction mixture) of Inorganic/ Org Salt of cadmium	Final Conce: (in sample mixture) of inorg/org Salts of cadmium	Inorganic Salt of cadmium (CNT)	Plasma GSH Control	Remaining %age of GSH	Inorganic salt of cadmuim (CNT)	Cytosolic fraction GSH Control	Remaining % age of GSH
0.0001mM	0.00005mM	0.003µM	3.20	4.53	70.64	2.99	4.99	59.92
0.001mM	0.0005mM	0.033µM	2.91	4.53	64.24	2.42	4.99	48.50
0.01mM	0.005mM	0.33µM	2.58	4.53	56.95	2.11	4.99	42.29
0.1mM	0.05mM	3.33µM	1.93	4.53	42.60	1.61	4.99	32.27
1.0mM	0.5mM	33.33µM	1.35	4.53	29.80	1.28	4.99	25.65
2.0mM	1.0mM	66.66µM	1.02	4.53	22.52	0.99	4.99	19.84

temperature indicating that human body temperature favors interaction between cadmium and GSH of blood plasma/cytosolic fraction during cadmium toxicities. The decrease in GSH of plasma from lowest to highest used concentration of cadmium nitrate tetra hydrate at 37°C was 29.36%, 35.76%, 43.05%, 57.40%, 70.20% respectively while the decrease in GSH of cytosolic fraction from lowest to highest used concentration of cadmium nitrate tetra hydrate at 37°C was 40.08%, 51.50%, 57.71%, 67.73%, 74.35%, 80.16% (table 2). Similarly table 3,4 shows the result of interaction of cadmium nitrate tetra hydrate and plasma /cytosolic fraction indicating that in both the compartments the decrease in GSH is more at 37°C than the corresponding other two used ranges of temperature during the experiment.

DISCUSSION

pH dependent effect shows greater depletion of GSH in buffer heaving pH near to physiological pH then the observed values of depletion of GSH in other phosphate buffers having pH 7.0,8.0 and 8.5.The depletion or drop in GSH level in both fractions (plasma/ cytosolic fraction) was less than in buffer heaving pH 7.5 (near to physiological pH), this indicates that metal- complex formation or oxidation of GSH into GSSG was fast in this pH and physiological pH favors the interaction between cadmium nitrate and GSH of these compartments during cadmium toxicities. These results have proved that physiological pH favor the interaction between heavy metals like cadmium. The scientific data of our study about the interaction between various concentrations of cadmium and GSH contents of plasma/ cytosolic fraction will enable us to understand the role of GSH in heavy metal detoxification and the need of chelation therapy during toxicity of such metals and also strengthen our knowledge about metal toxicity and role of GSH. If we compare the depletion of GSH contents in plasma and cytosolic fraction, we find that GSH level of cytosolic fraction has decrease much more than GSH level in plasma fraction part of whole blood for the effect of inorganic compounds of cadmium was promising and proved that inorganic compounds of Cd^{II} crossed the semi permeable membrane of red blood cells indicating sufficient penetrating power of these metals to RBCs. Our study suggest that toxicity of heavy metal under consideration is equally toxic deteriorating to RBCs and cytosolic GSH as to GSH content and other tissues and organs of human body. Hypothesis was that Cd^{II} metal causes modulation in the status of GSH either to glutathione disulfide (GSSG) or formation of $Cd\ (GS)_2$ complexe respectively. Our results support this hypothesis that interaction of cadmium with components of venous blood, results depletion in concentration of GSH in these components. GSH and other chelating agents are very important and necessary to protect the body during cadmium toxicities (Fowler, 1978). In different diseases

Table 3: Result of various concentrations of cadmium nitrate tetra hydrate (Final conce: 0.003-66.66μM) on the modulation and chemical status of plasma GSH

Parameters		0.003μM Conc:	0.03μM Conc:	0.33μM Conc:	3.33μM Conc:	33.33μM Conc:	66.66μM Conc:
pH	7.0	2.328	2.219	2.143	2.028	1.977	1.882
	7.5	1.844	1.761	1.672	1.578	1.500	1.410
	8.0	2.000	1.914	1.831	1.729	1.659	1.570
	8.5	2.143	2.054	1.971	1.869	1.800	1.710
Temperature (^{o}C)	25	2.359	2.258	2.200	2.066	1.977	1.901
	37	2.219	2.143	2.054	1.958	1.882	1.790
	45	2.302	2.207	2.156	2.035	1.971	1.901
Plasma GSH Control		2.914	2.914	2.914	2.914	2.914	2.914

Table 4: Result of various concentrations of cadmium nitrate tetrahydrate (Final conce: 0.003-66.66μM) on the modulation and chemical status of cytosolic fraction GSH

Parameters		0.003μM Conc:	0.03μM Conc:	0.33μM Conc:	3.33μM Conc:	33.33μM Conc:	66.66μM Conc:
pH	7.0	1.850	1.754	1.716	1.633	1.601	1.544
	7.5	1.359	1.300	1.245	1.181	1.124	1.073
	8.0	1.519	1.449	1.404	1.340	1.283	1.232
	8.5	1.659	1.589	1.544	1.480	1.423	1.372
Temperature (^{o}C)	25	1.875	1.800	1.767	1.672	1.601	1.563
	37	1.742	1.678	1.627	1.563	1.512	1.455
	45	1.818	1.735	1.722	1.640	1.710	1.547
Cytosolic GSH Control		2.971	2.971	2.971	2.971	2.971	2.971

like cerebral ischemia-reperfusion injury (Packer *et al.*, 2001), there are low levels of GSH which causes neurological damage (Nordberg, 1984). In our study the second influential factor was temperature, it was observed that depletion of GSH in these compartments was greater at 37°C, which is the temperature near to human body temperature showing that human body temperature provides a favorable environment for the interaction between cadmium compounds and GSH. The drop in GSH contents at other two temperatures, which were used in this experiment was less as compare to drop of GSH at 37°C.

A positive correlation was observed between the interaction of plasma GSH/cytosolic GSH to different concentration of CNT and GSH depletion with pH and temperature. Cadmium is classified as type one human carcinogen by International Agency for Research on Cancer of USA (IARC, 1993). The initiative of present study was that now a days there is an increased use of cadmium metal and ultimately it enters into food chain and has directly or indirectly an effect on human body. Tissue damages occurs during cadmium toxicities or chronic exposure to cadmium and these damages are due to thiol depletion and oxidative stress (Ercal *et al.*, 2001).

Cadmium is the cause of many complications, which arise due to lack of zinc and selenium as it replaces zinc and selenium inmetalloenzymes (Moriarty and Jones, 2004). Our results provide a scientific data that both pH and temperature are the influential factors, which favor the interaction between cadmium and GSH of plasma and cytosolic fraction during cadmium toxicities.

CONCLUSION

Our study shows that depletion of GSH of human blood plasma and cytosolic fraction is not only pH and temperature dependent but human body temperature and physiological pH also favors the depletion of GSH in these compartments during cadmium toxicities.

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