Cadmium-glutathione complex formation in human t-cell and b-cell lymphocytes after their incubation with organo-cadmium diacetate

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Abstract: Cadmium intake is associated with oxidative stress that causes depletion of intracellular as well as extra cellular reduced glutathione. There is strong evidence indicating that reactive oxygen species and reactive nitrogen species generated in the presence of cadmium could be responsible for its toxic effects in many cells and tissues. Depletion of reduced glutathione in various cells, especially in T and B-lymphocytes, causes extreme damage to the antioxidant defense system of body. The aim of this research work was to investigate the metabolic changes that occur in T and B lymphocytes after their incubation with organ cadmium diacetate by using Ellman's spectrophotometric method of thiol quantification. The results of the present study indicate that cadmium depleted T and B lymphocytes GSH to a harmful extent. It is proposed that this depletion is due to the bivalent cadmium glutathione complex formation, oxidation of reduced glutathione (GSH) to its oxidized form, or both.

Keywords: Lymphocytes, Cd-Glutathione, GSSG, oxidation-reduction, ROS.

INTRODUCTION

Gamma-glutamyl-cysteinyl-glycine is the reduced form of glutathione (GSH) and is the non-protein thiol molecule present in abundant within the cells (0.1mM to 10mM). The thiol moiety in glutathione molecule endows GSH with the property and potential to act as a reductant by donating an electron to endogenous acceptor as well as exogenous foreign particles that get entry into the body such as heavy metals in the form of pollutant of air, water, food or drugs. Thus, glutathione acts as a stabilizer of free radicals by giving a hydrogen atom to these endogenous and exogenous species (Burkitt, 2011; Ghezzi, *et al*., 2005). When transition metals like cadmium, arsenic, aluminum and mercury enter inside the human body through any source, the reduced glutathione promote a per oxidant effect by a metal reducing action (White *et al*., 2004; Khan H *et al.,* 2010, 2011a and 2011b; Muktiar *et al.,* 2013; Shah *et al.,* 2013 & 2013a; Khan J *et al.,* 2012).

Utilization of GSH in the detoxification of various metals causes perturbation of the crucial GSH: GSSG balance which is usually tightly regulated and crucial to the survival of cells (Klejdus *et al*., 2004). A deficiency of reduced form of glutathione exposes all cells to risk of oxidative damage (Droge, 2002). The urgent determination of GSH is particularly important due to high importance of GSH in homeostasis metabolism and in the detoxification of both essential metals like Zn, Mn, Cu and highly toxic heavy metals like Cd, As, Pb, Al etc (Vacek *et al*., 2004). The heavy metals ion such as Cd, Hg, As and Pb come into the whole environment from different sources and these sources may be natural and anthropogenic as a result these heavy metals ion enter into the plants and animals (Skrivan *et al*., 2006). Food chain is the main source of distribution of heavy metals and these heavy metals are accumulated in the predators including man (Zehnalik *et al*., 2004). Both non-essential heavy metals after their consumption by the organism and the heavy metals which as a toxic and foreign particles for the organism are covalently bound into biomolecules through sulfhydryl groups of cysteine residues (Petrlova *et al*., 2006). Large-scale industrial use of cadmium and its use in agricultural products have increased cadmium contamination in the soils and water. This should be a major global concern because cadmium is bioaccumulated in upper levels of the food chain (Satarug *et al*., 2010).

Along with occupational exposure, cigarette smoking and diet are the two main non-occupational exposure sources of cadmium (Satarug and Moore, 2004). Obviously cadmium levels in human body will continue to increase in the future and might lead to higher incidence of cadmium-related diseases such as cardiovascular disease, osteoporosis, hypertension, nephrotoxicity, diabetes, hepatotoxicity and cancer of various organs (Fowler, 2009). Decreased level of GSH is associated with all these diseases. Hence, it is necessary to analyze spectrophotometrically the interaction between cadmium and human T-cell and B-cell lymphocytes since decrease in GSH levels in T and B lymphocytes may play a big role in impaired function of these cells of human immune system while weak immune system results in different diseases.

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MATERIALS AND METHODS

Materials

The following reagents were sourced from the indicated suppliers: Roswell park memorial institute (RPMI)-1640 (Sigma), Ellman's reagent or 5,5-dithibis-2-nitrobenzoic acid (Sigma), L-glutathione (Fluka), Fetal calf serum (Sigma), Ficoll-paque plus (Sigma) and cadmium diacetate (Aldrich). All solutions were prepared in double refined distilled water on daily basis and the samples were flushed with high purity nitrogen and sometimes argon depending upon the availability of these gases. The rest of the chemicals and reagents were of analytical grade while the measurements were recorded on U.V-visible spectrophotometer.

Methods

Preparation of stock solutions

Stock solution of Ellman's reagent (1.0mM) was prepared by dissolving exactly 29.7mg of this reagent in 75.0ml of phosphate buffer (pH=7.6). Reduced glutathione (23.1mg) was added in 75ml of 0.2M phosphate buffer pH 7.6 in order to get 1.0mM GSH stock solution. Sodium chloride (90mg, pharmaceutical grade) was added in quantity sufficient of double refined distilled water to prepare 100 ml of 0.9% stock solution of NaCl. Four parts of fetal calf serum was mixed with 45 parts of RPMI-1640 to get a balance salt stock solution. Ficoll-paque plus was used as was purchased without further purification. Stock solution of organocadmium diacetate was prepared by dissolving 23.0mg of cadmium diacetate in sufficient distilled water to prepare 100ml of 2mM stock solution. Further dilutions of the organocadmium stock solution were prepared which were 0.0001mM, 0.001mM, 0.01mM, 0.1mM and 1.0mM.

Isolation of T-cell and B-cell lymphocytes

Ficoll-paque plus supplier's provided standard procedure and instructions were fully practiced to isolate T-cells and B-cells lymphocytes. A 2000µl aliquot of treated blood sample of a healthy human volunteer was carefully diluted with 2000µl of balanced salt solution of RPMI 1640 plus 10% fetal calf serum and layered on the ficoll-paque plus solution. The resultant two-phase system was centrifuged at 400xg for 35 minutes at a temperature of 19^{o}C. Five different layers were obtained on centrifugation based on their densities. Plasma was the top most layer, while the bottom-most layer was of erythrocytes (because of high density erythrocytes layer was completely sedimented at bottom of the tube). Granulocytes settled immediately above the erythrocytes layer. Due to low density than erythrocytes and granulocytes, the lymphocytes remained suspended in the form of layer with platelets at the interface between the plasma (upper most layer) and the ficoll-paque plus layer.

The lymphocytes were recovered from the interface and were washed with RPMI-1640 in order to remove any traces of ficoll-paque plus and plasma. 20% sucrose gradient layer over ficoll-paque plus was used to remove all the traces and contamination of platelets. The lymphocytes because of high density than platelets, remained sedimented on ficoll-paque plus while the platelets were left on the top of the sucrose gradient layer.

Separation of T-cell and B-cell lymphocytes

Isolated lymphocytes were further separated in T-cells and B-cells. For separation of T-Cells, an upright column was prepared using 1ml syringe containing nylon wool, which was already incubated for 30 minutes at 37^{o}C. The lymphocytes sample was slowly poured in this column and after a few minute T-cells were collected below the column in the form of drops in a test tube. By adding slowly 10ml of RPMI to the column, the remaining T-cells were also obtained in the test tube below the column.

Because of irregular shape of B-cells, they had stuck to the nylon wool in the above mentioned column thus by adding 5-10ml RPMI to this column, B-cells were drop wise collected in another test tube by squeezing nylon wool slowly with glass rod in the column. As a result of squeezing process, drops from the column had started following in the test tube, which were carefully collected till to the last drop and these collected drops contained B-cells. Now 50µl HCl (0.1N) was added to both the test tubes to keep glutathione or T-cells and B-cells GSH in intact form. Both the test tubes containing T and B-cells were tightly sealed and kept on ice till further use.

T-Cells control

The T-cell control was prepared by mixing the isolated T-cells fraction and 0.9% NaCl solution in the ratio 1: 1, but without adding organocadmium diacetate.

B-cells control

The B-cell control was prepared by mixing the isolated B-cells fraction and 0.9% NaCl solution in the ratio 1:1, but without adding organocadmium diacetate.

Experimental design

After the process of centrifugation, carefully, plasma and cytosolic fraction were collected in separate sample tubes. Concentration and time dependent effect of cadmium diacetate (organo-cadmium) on the chemical status and modulation of both T & B cells lymphocytes was studied. The used concentrations of cadmium diacetate in reaction mixture were 0.0001mM, 0.001mM, 0.01mM, 0.1mM, 1.0mM and 2.0mM while the final concentrations of cadmium diacetate were 0.003µM, 0.03µM, 0.33µM, 3.33µM, 33.33µM and 66.66µM respectively. Analysis of interaction between cadmium diacetate and T-Cells GSH and B-cell GSH at different times of incubation between the two was also studied and the different times of incubation were 0 minute, 20, 40, 60, 90 and 120 minutes.

Experimental procedure

1.T-cells and B-cells fraction of lymphocytes were treated with different concentrations of cadmium diacetate.

2.The effect of cadmium diacetate on the T and B cells glutathione levels at 0, 20, 40, 60, 90 and 120 minutes was analyzed in order to estimate the effect of organo-cadmium on GSH levels as a marker of cellular toxicity.

Estimation of the concentration of GSH

Ellman's method (Ellman, 1959) was used for the determination of level of GSH. Six samples of isolated T-cells and B-cell separately were prepared and to each sample of T-cells and B-cells different concentrations of cadmium diacetate were mixed in such a way that 6 samples of isolated T-cells were prepared, to each these 6 sample 6 different concentration of cadmium diacetate were added and in the same way a set of 6 other samples of isolated B-cells were prepared, to each these B-cells sample, 6 different concentration of cadmium diacetate were added, both the sets of 6 samples (T-cells + different concentrations of cadmium diacetate and B-cells + different concentrations of cadmium diacetate) were incubated for 10 minutes and after 10 minutes, 0.2ml from each T-cells + cadmium diacetate sample as well as from each B-cells plus cadmium diacetate was taken and mixed with 23ml of phosphate buffer following by the addition of 0.5ml of Ellman's reagent and were incubated for 5 minutes. After 5 minutes absorbance of each sample was recorded at fixed wavelength λmax: 412 nm against T-cells and B-cells control respectively. The obtained absorbance was converted into concentration of GSH by linear regression equation (Y=m c + b).

Standard curve

Ellman's method was followed to prepare standard curve of GSH by using different concentrations of reduced glutathione (fig. 1).

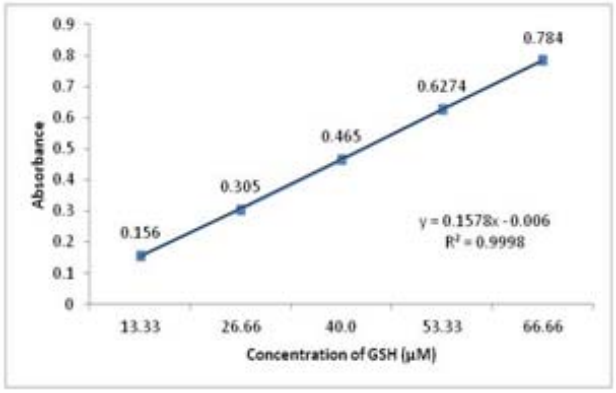

Fig. 1: Standard curve of GSH.

RESULTS

Concentration dependent effect of cadmium diacetate on T-cells GSH status

Various aqueous solutions of cadmium diacetate were prepared and in these dilutions final concentration of cadmium diacetate was in the range of 0.003µM to 66.66 µM (table 1). These different concentrations were treated with T-cells lymphocytes to determine the effect of organocadmium on T-cells GSH. Our analysis shows that cadmium diacetate in its lowest used concentration has dropped GSH level in T-cells significantly ($p<0.001$) as the GSH drop by this concentration was 18.66% with respect to T-cells GSH control while the depletion by second used concentration of cadmium diacetate was 21.79% and then 24.27%, 30.49%, 33.39% and 35.01% respectively when compared to T-cells GSH control as shown in fig. 2.

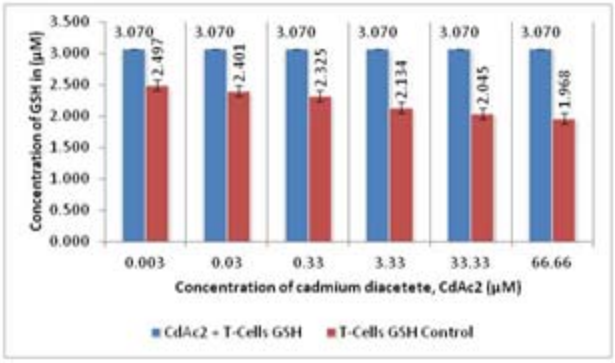

Fig. 2: Effect of different concentrations of cadmium diacetate ($CdAc_2$) on the chemical status of T-lymphocytes-GSH level (concentratin effect). T-lymphocytes-GSH control (1: 1, 1000µl blood: 1000µl 0.9% normal saline solution). Results aore the mean ±SE of 3 experiments of T-lymphocytes fraction.

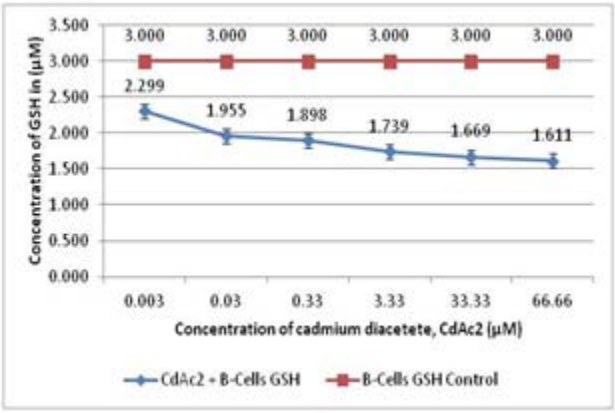

Fig. 3: Effect of different concentrations of cadmium diacetate ($CdAc_2$) on the chemical status of B-lymphocytes-GSH level (concentration effect). B-lymphocytes-GSH control (1:1 1000µl blood: 1000µl 0.9% normal saline solution). Results are the mean ±SE of 3 experiments of T-lymphocytes fraction.

Time dependent effect of cadmium diacetate on T-cells GSH status

It was also a matter of interest to estimate the drop or decrease in GSH level of T-cells with the increasing time of incubation of cadmium diacetate and T-cells. It was observed that by increasing the time of incubation, there was further significantly ($p<0.001$) decrease in GSH level

of T-cells. Lowest used (0.003µM) final concentration of cadmium diacetate has decreased GSH level in 0 minutes to 120 minutes about 18.66 % as compare to T-cells control while other concentrations have depleted GSH 29.25%, 35.47%, 40.46%, 43.78% and 46.19% respectively with respect to T-cells GSH control (table1). The comparison between the lowest and highest used concentrations of cadmium diacetate on T-lymphocytes GSH is shown in fig. 4. The effect of pH and temperature (table1) on the interaction between cadmium diacetate and T-lymphocytes GSH is fast and depletion in GSH is more in these cells at pH 7.5 and 35°C respectively which are both near to physiological pH and temperature.

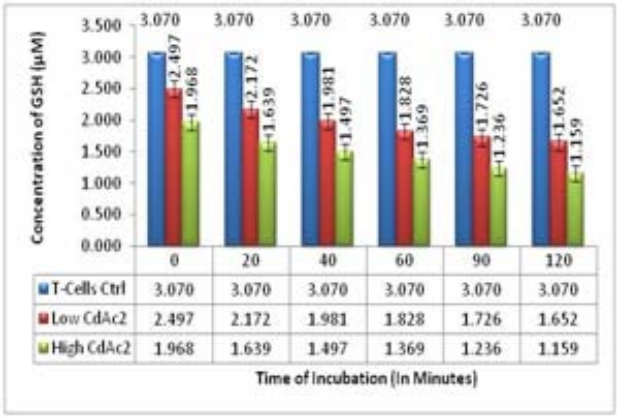

Fig. 4: Effect of cadmium diacetate ($CdAc_2$) on T-lymphocytes-GSH with time of incubation from 0 min: to 120min: Control (1000µl of blood: 1000µl of 0.9% normal saline 1:1) Effect of lowest used $CdAc_2$ concentration (0.0001mM) Effect of highest used $CdAc_2$ (66.66mM). Results are the mean ± SE of 3 experiments of T-lymphocytes- GSH.

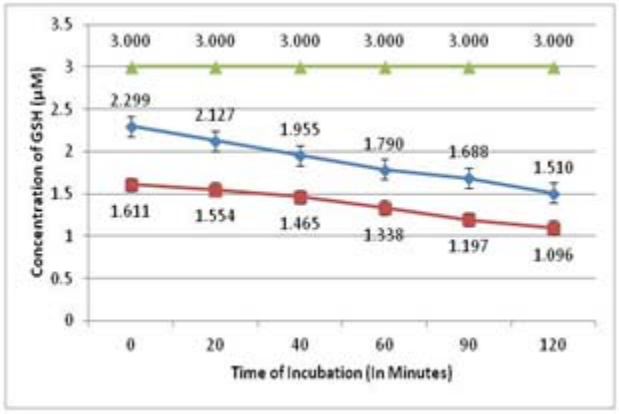

Fig. 5: Effect of cadmium diacetate ($CdAc_2$) on B-lymphocytes-GSH with time of incubation from 0 min: to 120 min: Control (1000µl of blood: 1000µl of 0.9% normal saline 1:1) Effect of lowest used $CdAc_2$ concentration (0.0001mM) Effect of highest used $CdAc_2$ (66.66mM). Results are the mean ±SE of 3 experiments of B-lymphocytes- GSH.

Concentration dependent effect of cadmium diacetate on B-cells GSH status

Different aqueous dilutions of cadmium diacetate having the final concentration of cadmium diacetate 0.003µM, 0.03µM, 0.3µM, 3.33µM, 33.33µM and 66.66µM were treated with isolated B-cells lymphocytes in order to determine the effect of cadmium diacetate on GSH level of B-cell and it was found that there is significantly ($p < 0.001$) drop in B-cells GSH level indicating that cadmium diacetate has profound effect on B-cells GSH contents. The lowest used concentration of cadmium diacetate has depleted GSH to 23.37% as compare to B-cells control solution. The other concentrations of cadmium diacetate in order of increasing used micromoles has depleted B-cells GSH contents to 34.83%, 36.73%, 42.03%, 44.37% and 46.30% with respect to B-cells GSH control respectively, as shown in fig. 3.

Time dependent effect of cadmium diacetate on B-cells GSH status

After knowing the effect of cadmium diacetate on B-cells GSH contents, it was very important to analyze the effect of increasing time of incubation between cadmium diacetate and B-cells GSH contents. The time intervals were 0, 20, 40, 60, 90 and 120 minutes and it was found that as the time passes, there is further significantly ($p < 0.001$) decrease in B-cells GSH contents and there exist a co-relation between time of incubation and in decrease of B-cells GSH contents within 120 minutes in order of lowest used concentration to highest used concentration of cadmium diacetate. The drop in B-cells GSH contents level by lower used concentrations of organo-cadmium i-e cadmium diacetate from 0 to 120 minutes was 23.37%, 29.10%, 34.83%, 40.33%, 43.73% and 49.67% respectively (table2). The effect of lowest and highest used concentrations of cadmium diacetate on B-cells GSH contents is shown in fig. 5. The effect of pH and temperature (table 2) on the interaction between cadmium diacetate and B-lymphocytes GSH is fast and depletion in GSH is more in these cells at pH 7.5 and 35 °C respectively which are both near to physiological pH and temperature.

DISCUSSION

Functions of T-cells depend upon the level of T-cells GSH. Depletion of GSH suppresses T-cells activation (Potter, 1997), proliferation (Taylor *et al*., 1997), cytokine production (Peterson *et al*., 1998) and generation of cytotoxic T lymphocytes responses (Gmunder *et al*., 1991). As shown in table 1 and fig. 2, it is clear that cadmium is depleting T-cells GSH levels that means cadmium is the cause of suppression of T-cells dependent immune responses as these responses are T-cells GSH contents dependent, and maintenance of T-cells GSH level is needed for normal immune responses. Insufficient GSH leads to a preferential decrease in frequency of responding

Table 1: Results of various concentrations of cadmium diacetate on the modulation and chemical status of T-lymphocytes with time (After separation)

Parameters			0.003			0.03	0.33	3.33	33.33	66.66
Time (Minutes)			Conc:			Conc:	Conc:	Conc:	Conc:	Conc:
1	Remaining conc: of GSH at 0 mint		2.497			2.401	2.325	2.134	2.045	1.968
		pH		7.0	2.363	2.255	2.178	2.064	2.019	1.917
				7.5	1.879	1.796	1.707	1.611	1.535	1.446
				8.0	2.032	1.955	1.866	1.764	1.701	1.605
				8.5	2.172	2.096	2.006	1.904	1.847	1.745
		Temperature (^{o}C)		25	2.395	2.293	2.229	2.102	2.025	1.981
				37	2.255	2.178	2.089	1.994	1.917	1.828
				45	2.338	2.248	2.191	2.070	2.006	1.943
2	Remaining conc: of GSH at 20 mint		2.172			2.051	1.968	1.790	1.713	1.639
3	Remaining conc: of GSH at 40 mint		1.981			1.892	1.796	1.624	1.548	1.497
4	Remaining conc: of GSH at 60 mint		1.828			1.752	1.669	1.497	1.420	1.369
5	Remaining conc: of GSH at 90 mint		1.726			1.637	1.573	1.382	1.318	1.236
6	Remaining conc: of GSH at 120 min		1.652			1.586	1.516	1.306	1.242	1.159
	T-lymphocytes GSH Ctrl		3.070			3.070	3.070	3.070	3.070	3.070

Table 2: Results of various concentrations of cadmium diacetate on the modulation and chemical status of B-lymphocytes with time (After separation)

Parameters			0.003			0.03	0.33	3.33	33.33	66.66
	Time (Minutes)		Conc:			Conc:	Conc:	Conc:	Conc:	Conc:
1	Remaining conc: of GSH at 0 mint		2.299			1.955	1.898	1.739	1.669	1.611
		pH		7.0	1.892	1.790	1.752	1.669	1.637	1.580
				7.5	1.395	1.331	1.280	1.217	1.159	1.108
				8.0	1.554	1.484	1.446	1.376	1.318	1.268
				8.5	1.694	1.624	1.586	1.459	1.408	1.344
		Temperature (^{o}C)		25	1.911	1.828	1.803	1.707	1.637	1.599
				37	1.777	1.713	1.662	1.599	1.548	1.490
				45	1.854	1.771	1.758	1.675	1.637	1.592
2	Remaining conc: of GSH at 20 mint		2.127			1.898	1.860	1.707	1.611	1.554
3	Remaining conc: of GSH at 40 mint		1.955			1.771	1.688	1.541	1.478	1.465
4	Remaining conc: of GSH at 60 mint		1.790			1.669	1.554	1.446	1.401	1.338
5	Remaining conc: of GSH at 90 mint		1.688			1.516	1.459	1.357	1.299	1.197
6	Remaining conc: of GSH at 120 mint		1.510			1.427	1.344	1.255	1.178	1.096
	B-lymphocytes GSH Ctrl		3.000			3.000	3.000	3.000	3.000	3.000

cells, with T-cells more sensitive than B-cells. In the present study, we have determined the effect of various concentrations of cadmium diacetate on the chemicals status of T-lymphocytes and B-lymphocytes and also the effect of duration of incubation of cadmium on the levels of GSH of lymphocytes. Absorbance of each sample was recorded and was converted into concentration of GSH. There was decrease in T-lymphocytes GSH contents even with the minimum used concentration of organo-cadmium, there is a lot of depletion in reduced glutathione concentration of T-cells and with the increase in concentration of cadmium diacetate, there was further drop in GSH contents of T-cells. In case of B-lymphocytes, the depletion in their GSH contents was comparatively more than depletion in GSH contents of T-lymphocytes and the same effect of increasing concentration of organo-cadmium on B-cells GSH contents was observed as in case of T-cells. These changes in T-cells table 1, fig. 2,4 and B-cells table 2, fig. 3,5 shows statistical significant decrease in GSH contents of both types of lymphocytes suggesting and supporting the already published data that decrease in GSH level of these two types of lymphocytes impair their functions and have cause suppression of immune responses resulting in weaken immune and antioxidant defense system of body ultimately leading to death of cells. In various studies it has been demonstrated that there is a co-relation between decreased GSH in T-cells and increase apoptosis (Chiba *et al*., 1998).

Heavy metals including cadmium, mercury, arsenic and lead are covalently bound into bio-molecules through sulfhydryl (-SH) groups of cysteine residue. The same finding report (Hashmat *et al*., 2012) that cadmium has

depleted GSH level in aqueous media by increasing the concentration and time of incubation has confirm this study. Keeping in view the above discussion it seems that the possible cause of depletion of GSH contents of T & B lymphocytes is due to interaction between Cd^{+2} and -SH group of GSH of these cells or this decrease is due to the conversion of GSH into GSSG (oxidized form of glutathione). The possibility of formation of other intermediates cannot be ignored but it was not possible to find these intermediates exactly under the given experimental conditions and the exact mechanism of action is unknown.

$$Cd(CH_3COO)_2 \longrightarrow Cd^{2+} + 2CH_3COO^{-1}$$
$$Cd^{2+} + 2GSH \longrightarrow Cd(SG)_2 + 2H^{+}$$
$$GS^{-1} + GS^{-1} \longrightarrow GSSG$$

The decrease/ depletion of GSH contents of T and B lymphocytes indicate that along with other harmful effects of cadmium, it also suppresses the activation and functions of these cells suggesting the industries to have a check on the excessive use of cadmium compounds in agricultural products, cadmium- nickel batteries, PVC pipes factories and as an additive in cigarette manufacturing. Cellular GSH concentration is intimately associated with cellular damage that can results from oxidative stress due to metals like cadmium, mercury, arsenic, lead etc. Lymphocytes activation is sensitive to GSH concentration and changes in concentration of GSH specially their function goes to declination in case of depletion of their GSH contents leading to their impair function and finally to their death. B-lymphocytes are primarily responsible for producing antibodies and these cells are regulated by T-lymphocytes and microphage.

CONCLUSION

So we conclude that immunological alterations occur which are associated with GSH contents of T-cells and B-cells and hence suppression in lymphocytes activity is co-related with depletion in GSH level of these cells which ultimately with the increase in cadmium concentration causes further decrease in GSH level leading to cells death.

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