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Cisplatin-induced Toxicological Effects in Relation to the Endogenous Tissue Glutathione Level in Tumor-Bearing Mice

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Abstract : Cisplatin treatment of Dalton's lymphoma-bearing mice for five days induced toxicological effects in kidney, testes and blood. The development of tubular dilation, degeneration, necrosis of epithelial cells and lining membrane damage in kidney and vacuolated, deranged and broken seminiferous tubules in testes have been observed. In blood, cisplatin treatment caused a reduction in red blood cells, white blood cells, and hemoglobin. Cisplatin treatment of mice caused a significant decrease of glutathione levels in kidney, blood and Dalton's lymphoma (DL) cells. The cisplatin-induced toxicity was further enhanced in all the tissues when buthionine sulfoximine, an inhibitor of glutathione synthesis, was used in combination with cisplatin. The catalase enzyme activity was very sharply reduced (60-70 %) in tissue as well as DL cells. The observed decrease of GSH level accompanied with the reduced catalase activity after cisplatin treatment may hamper antioxidant machinery in the cells and contribute to the development of tissue toxicity and DL cytotoxicity during cisplatin mediated chemotherapy in the hosts.

Key Words : Cisplatin, Toxicity, Glutathione, Catalase, Kidney

Introduction :

Cis-diamminedichloroplatinum-(II), commonly known as cisplatin, is an effective anticancer drug used for the treatment of a wide spectrum of malignancies (Rosenberg, 1985; Prasad and Giri, 1994; Go and Adjei, 1999). The major cytotoxic target of cisplatin in a cell is suggested to be the DNA with the formation of intra- as well as inter strand cross-links which could be the main mechanism behind its anticancer activity (Coste *et al.*, 1999; Kartalou and Essigmann, 2001a). In addition to DNA in the cells, cisplatin's interaction with phosphatidylserine in membranes (Speelmans *et al.*, 1997) and ability to affect host immune response (Collins and Kao, 1989), cell surface (Prasad and Sodhi, 1981), tissue calcium and potassium levels (Prasad and Giri, 1999), various enzymes such as 5'-nucleotidase, arginase, cathepsins, lactate dehydrogenase (Prasad *et al.*, 1999), mitochondria (Kharbangar *et al.*, 2000) and some glutathione related enzymes (Khynriam and Prasad, 2002) have been reported and thereby suggested that these effects should also contribute as additional components in the mechanism of anticancer activity of cisplatin.

The major limitation in the full effective use of cisplatin against cancers is the development of several dose-limiting side effects, which include ototoxicity, nephrotoxicity (Krakoff, 1979), embryotoxicity (Keller and Aggarwal, 1983), peripheral neuropathy (Hamers *et al.*, 1991) and mutagenicity (Khynriam and Prasad, 2003a). Another limitation to the

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better success of cisplatin therapy is acquired drug resistance by cancer cells (Kartalou and Essigmann, 2001b). In an attempt to overcome these impediments in the host, the use of some modulating agents (Borch and Markman, 1989; Giri *et al.*, 1998) and/or new platinum analogues (Christian, 1992) have been tried with varying degree of success.

Glutathione (a tripeptide, L-g-glutamyl-L-cysteinyl-glycine), the major intracellular thiol and antioxidant, is involved in a variety of biological processes (Wang and Ballatori, 1998). Reduced glutathione (GSH) plays an important role in the synthesis of proteins and DNA, transport, enzyme activity, metabolism and protection of cells (Wang and Ballatori, 1998; Meister and Anderson, 1983). Catalase (*EC.1.11.1.6*), a wide spread enzyme in mammals (Tew, 1994), protects cells from oxidative damage by catalyzing decomposition of hydrogen peroxide (H_2O_2) to water and molecular oxygen (Ueda *et al.*, 1990). L-buthionine-(*S,R*)-sulfoximine (BSO) is an inhibitor of g-glutamyl cysteine synthetase (Griffith and Meister, 1987) and has been used to deplete cellular GSH. The possible mechanism of cisplatin-induced toxicity in relation to cellular GSH and catalase has not been known.

Therefore, the present studies were undertaken to investigate cisplatin-induced histological changes as well as GSH levels and catalase activity in tissues. Kidney, the primary organ for cisplatin excretion and testes, the source of male germ cells and blood, a medium for drug inter-organ transportation were specifically chosen for these evaluations.

Materials and Methods :

Animals and Tumor maintenance : The inbred Swiss albino mice colony is being maintained under conventional laboratory conditions keeping 5-6 animals in a propylene cage, using paddy husk as bed at 24-25^{0}C. The animals were fed with commercially available food pellets diet (Amrut laboratory, New Delhi) and tap water *ad libitum*. Ascites Dalton's lymphoma (DL) is being maintained *in vivo* in inbred Swiss albino mice by serial intraperitoneal (i.p) transplantation of about $1x10^7$ viable tumor cells in 0.25 ml of phosphate buffered saline (PBS, pH 7.4) per animal. Healthy male mice in the age group of 12-13 weeks were used for the experiment. Tumor-transplanted animals usually survived for 19-21 days.

Drug treatment : Therapeutic dose of cisplatin against malignant tumors has been established to be 8-10 mg/kg body weight (Rosenberg, 1985; Prasad and Giri, 1994). On the 10th day post-tumor transplantation, i.e. mid phase of tumor growth, mice were administered with single dose of cisplatin (8 mg/Kg body weight). After 1 to 5 days of cisplatin treatment (*i.e.* on the 11th, 12th, 13th, 14th and 15th day after tumor transplantation), kidney, testes, Dalton's lymphoma and blood were collected and used for GSH estimation. Control mice were injected with isotonic NaCl (0.89%) only.

GSH estimation : Tissue GSH level was determined following the method of Sedlak and Lindsay (1968). Briefly, cells pellet/tissue was weighed and its 5% homogenates were prepared in 0.02M EDTA, pH 4.7 using a motor-driven Teflon-pestle homogenizer (Metrex, India). To the 100ml homogenate or pure reduced form of

glutathione, 0.2 M Tris-EDTA buffer (1.0 ml, pH 8.2), 0.02M EDTA, pH 4.7 (0.9 ml) and Ellman's reagent (20ml) were added. After 30 min of incubation at room temperature, the reaction mixture was centrifuged and the absorbency of clear supernatants was read against a reagent blank (without sample) at 412 nm in a Beckman DU-640 spectrophotometer.

Determination of hematological parameters : For the evaluation of various blood parameters, blood was collected from the tail vein into a sterilized glass tube containing heparin (15-20 IU/ml blood) and the study was made on the same day. Red blood cell (RBC) count and hemoglobin (Hb) content were analyzed following the criteria of Dacie and Lewis (1975). White blood cells (WBC) count was done following the method of Swarup *et al.*, (1981). Blood GSH was determined according to Beutler *et al.,* (1963).

Histopathological determination : On the 10^{th} day post-tumor transplantation, animals were separated into four groups, consisting of 5-6 mice in each group. Mice in group I and II were administered with a single therapeutic dose (8 mg/kg body weight, i.p.) and sub-therapeutic dose (4mg/kg body weight, i.p.) of cisplatin respectively. As the maximum depletion (~ 45%) of GSH in DL cells has been observed at 8 h after BSO treatment (5mM/Kg body weight, i.p.) *in vivo* (Khynriam and Prasad, 2003), mice in group III and IV were treated with BSO 8 h prior to the treatment with cisplatin, 4mg/kg body weight and 8mg/kg body weight respectively. Control group of mice was injected with the same volume of 0.89% NaCl. After five days following cisplatin treatment, the animals from each group were sacrificed; kidneys and testes were collected, fixed in cold Bouin's fixative and processed for histological evaluation. Paraffin sections of 5 µm thickness were cut, stained with haematoxylin-eosin, examined and photographed under MEIJI microscope. Mean body weight changes and histological changes in kidney and testes of the mice under different experimental conditions were determined.

Catalase assay: Catalase (EC 1.11.1.6) activity was assayed in kidney and DL cells according to Aebi (1984). The same tissue supernatant used for enzyme assay was also used for protein estimation following the method of Lowry *et al.,* (1951) and enzyme specific activity was calculated by dividing the enzyme units by the milligrams of protein in the assay tissue supernatant. The assay volume (3.0 ml) contained 20 ml tissue supernatant, 1.98 ml of 50 mmol/L phosphate buffer (pH 7.0). Adding 1.0 ml of 30 mmol/L H_20_2 started the reaction maintained at 20^0C. The decrease in absorbency at 240 nm was monitored for 60 seconds in a Beckman DU-640 spectrophotometer. The enzyme activity was calculated using the extinction coefficient of 0.00394 L $mmol^{-1}$ mm^{-1} and the unit of enzyme activity was expressed as mmoles of H_2O_2 decomposed per minute.

The significance of changes in treated groups was compared with respective control using paired student's t-test. $P \leq 0.05$ was considered statistically significant.

Results :

As compared to the control, slight decrease of body weight in the treated groups was observed from the second day

after cisplatin (4 mg or 8 mg/Kg body weight) administration. The decrease in body weight was more pronounced (9-13 % with 4 mg and 17-20% with 8 mg cisplatin) by the 5th day of treatment.

The sections of kidney from control mice exhibit a clear, normal, zonal variation extending from the cortex to the medulla with usual tubules having intact epithelial cells (Figure 1; A ,B). Cisplatin treatment of mice with subtherapeutic dose (4 mg/Kg body weight) caused a moderate tubular dilation and disorganized epithelial cells (Figure 1; C), while, the treatment with therapeutic dose (8 mg/Kg body weight) showed more tubular damage with the formation of atrophied tubules and less intact epithelial cells (Figure 1; D).

Cisplatin treatment of tumor-bearing mice resulted damage in testes also indicating gonadal toxicity. The histological observation of testes from untreated, control mice showed normal pattern of outer germinal epithelium, seminiferous tubules and interstitial cells in between the tubules (Figure 2; A, B). Here also, like that of kidney, increased damage and derangement to testicular cells were noticed with higher dose of cisplatin treatment. Single subtherapeutic dose (4 mg/Kg body wt.) of cisplatin caused a slight derangement in spermatogonial mass (Figure 2; C) while at therapeutic dose (8mg/Kg body wt.) vacuolated and broken seminiferous tubules were observed repeatedly (Figure 2; D).

Cisplatin treatment of tumor-bearing mice caused a significant reduction in GSH level in kidney, blood and DL cells (Table 1), and also a decrease in various hematological parameters i.e. hemoglobin, RBC and WBC (Table 2).

As compared to cisplatin alone, the combined treatment of mice with BSO plus cisplatin results further decrease in blood GSH level as well as various hematological parameters. However, as compared to the treatment with cisplatin alone or BSO plus cisplatin, the combined treatment with cysteine plus cisplatin recovered the blood GSH level as well as various hematological parameters to almost control level (Fig. 3).

The activity of catalase sharply decreased in kidney and DL cells after cisplatin treatment (Fig. 4).

Discussion :

It has been reported that the sequence of cisplatin-induced renal toxicity is normal during the first 48 h, but thereafter, a decrease of both renal blood flow and glomerular filtration can be observed (Winston and Safirstein, 1985) with a maximum serum urea concentration occurring at 5-6 days of treatment (Kharbangar, *et al.*, 2000). Therefore, the evaluation of cisplatin-induced histo-toxicological effects on kidney and testes was executed after five days of treatment. It revealed a dose dependent nephrotoxic and testicular detrimental effects of cisplatin in the host. As compared to the control, decrease of body weight in the treated groups was also observed from the second day after cisplatin (4 mg or 8 mg/Kg body weight) administration and it became more pronounced (9-12 % with 4 mg and 17-20%

Table 1 : **GSH levels in the tissues of ascites Dalton's lymphoma-bearing mice after cisplatin treatment**.

Treatment	Tissue			
	Kidney (µmol/g)	Testes (µmol/g)	DL (µmol/g)	Blood (mg/ml)
Control	8.50 ± 1.22	9.28 ± 0.15	4.78 ± 0.26	0.64 ± 0.10
Cisplatin (24 h)	6.04 ± 0.42*	9.03 ± 0.29	3.51 ± 0.32*	0.57 ± 0.02
Cisplatin (48 h)	6.52 ± 0.37*	9.08 ± 0.23	3.81 ± 0.21*	0.47 ± 0.06*
Cisplatin (72 h)	6.94 ± 0.22*	9.10 ± 0.33	4.02 ± 0.23*	0.40 ± 0.01*
Cisplatin (96 h)	7.68 ± 0.33	9.11 ± 0.39	4.56 ± 0.45	0.42 ± 0.08*
Cisplatin (120 h)	7.05 ± 0.23	8.90 ± 0.28	4.16 ± 0.27	0.39 ± 0.07*

Control = Tissues from untreated tumor-bearing mice. DL = Dalton's lymphoma cells. Results are expressed as Mean ± S.D. Student's t-test, n = 4-5, as compared to control, *$P \leq 0.05$.

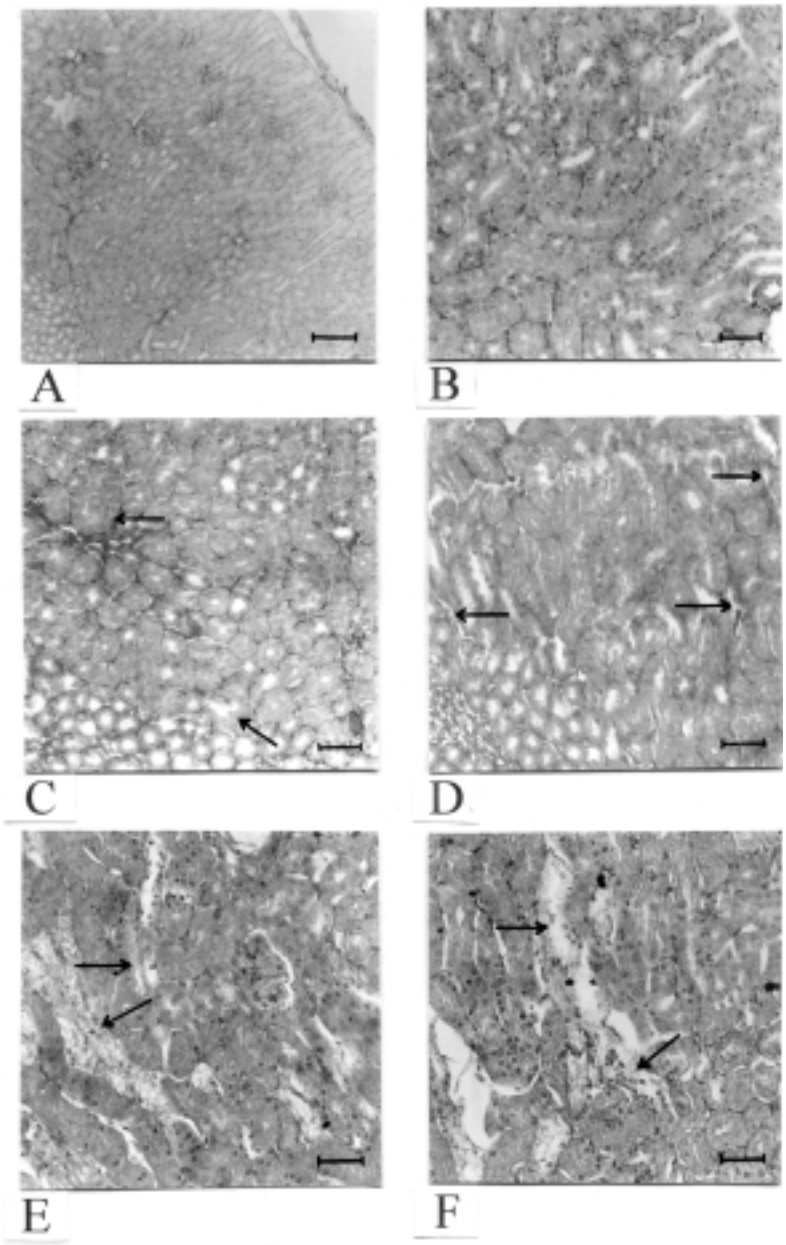

Fig. 1 : Histological observation of kidney from mice under different experimental conditions. **A**: Control, showing normal zonal variation extending from cortex to medulla. **B**: Higher power view of a portion of 'A' showing normal tubular distribution. **C**: After 5 days of cisplatin treatment (4 mg/kg body weight), tubules appear as slightly dilated (arrows). **D**: Therapeutic dose of cisplatin treatment (8 mg/kg body weight) for 5 days results in the appearance of many atrophied tubules (arrows) indicating necrosis. **E**: Combination treatment with BSO and cisplatin (8 mg/kg body weight) shows degenerated and damaged lining membrane tubules (arrows). **F**: Combination treatment with BSO and subtherapeutic dose of cisplatin (4 mg/kg body weight) showed lesser tubular damage as compared to that of therapeutic dose of cisplatin. Scale bar A= 100µ and B-F= 40µ.

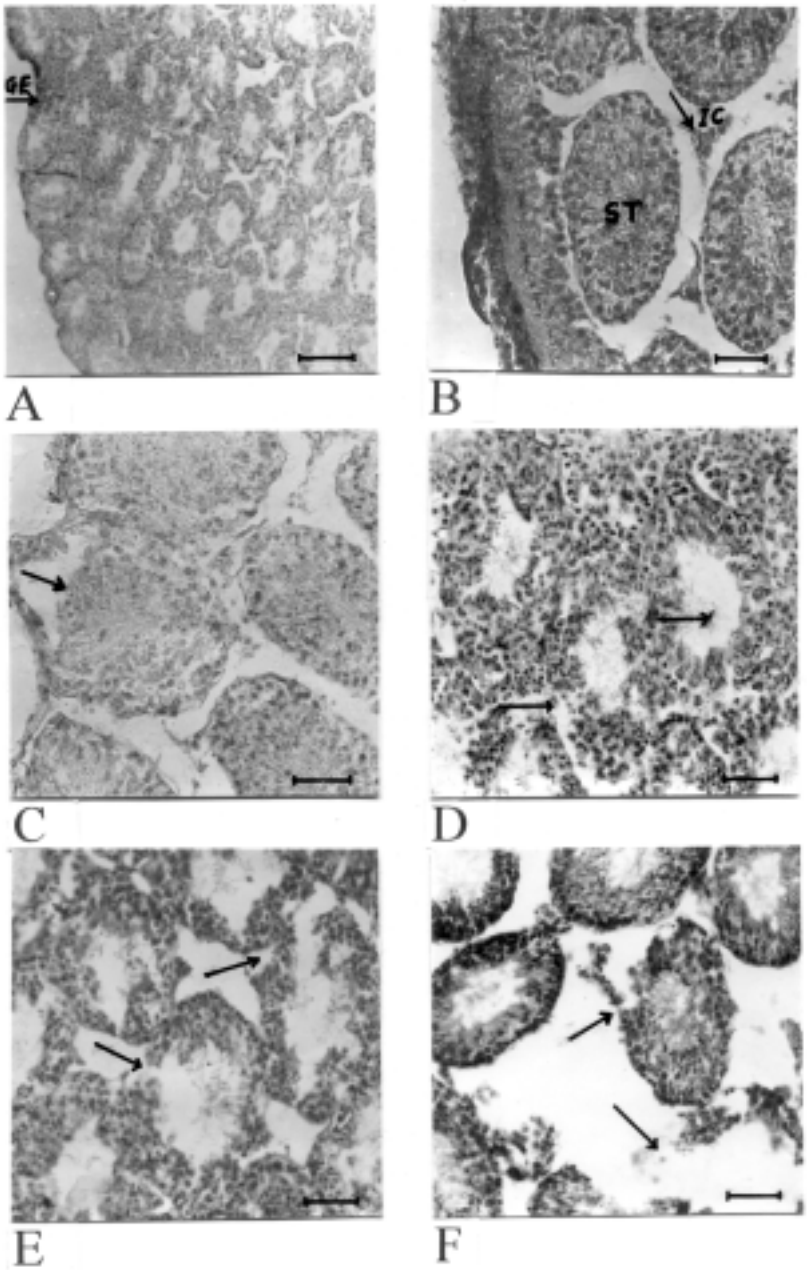

Fig. 2 : Histological pattern of testes from mice under different experimental conditions. **A:** Control, showing normal morphology and arrangement of germinal epithelium (GE), seminiferous tubules (ST) and interstitial cells (IC). **B:** Enlarged view of a portion of "A". **C:** After 5 days of cisplatin (4 mg/Kg body weight) treatment, showing a slight deranged spermatogonial mass (arrow). **D:** Higher dose of treatment (8 mg/kg body weight) of cisplatin results the formation of vacuolated seminiferous tubules (arrows). **E:** Combination treatment with BSO plus cisplatin (8mg/kg body weight) causes breaking of tubules (arrows). **F:** Combination treatment with BSO plus cisplatin (4mg/kg body weight) causing slightly more damage to tubules (arrows) as compare to cisplatin alone, shown in C. Scale bar A= 100µ and B-F= 40µ.

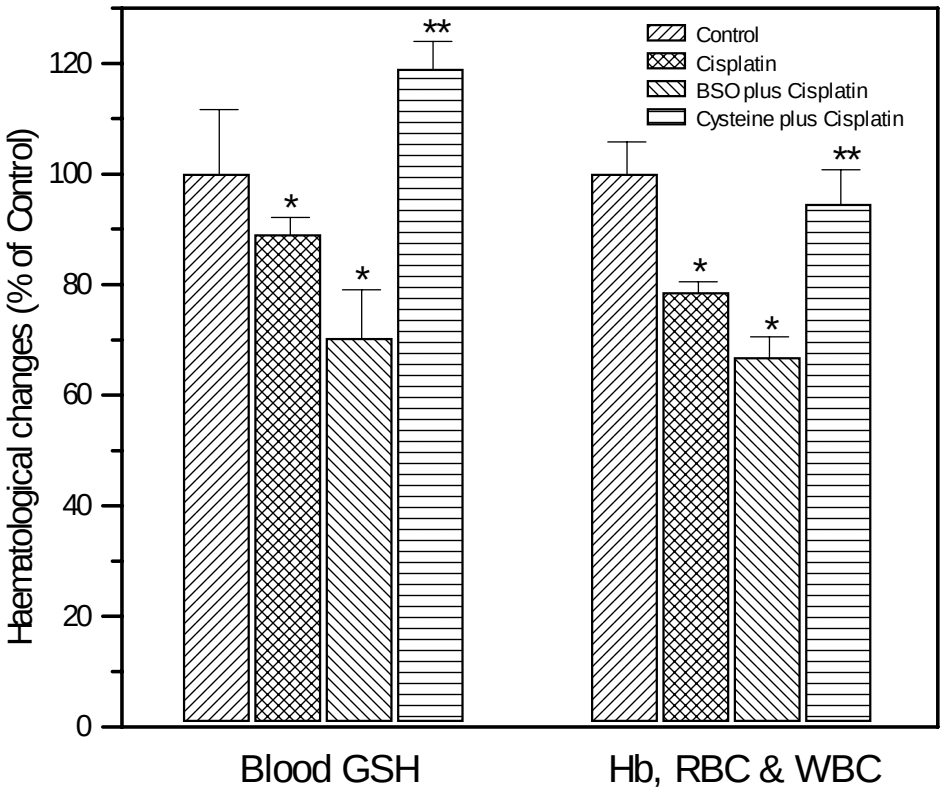

Fig. 3 : Histogram showing comparative percent changes in blood GSH level and combined hematotoxicological parameters after cisplatin, BSO plus cisplatin or cysteine plus cisplatin treatment condition. The data has been compared at corresponding time point (24 h) and dose (8 mg/Kg body weight) of cisplatin treatment in all the conditions. Student's t-test, n = 3-4, as compared to cisplatin alone (*) and respective BSO plus cisplain treatment (**), $^{*, **}$ $P \leq 0.05$.

Table 2 : Hematological changes in tumor-bearing mice following cisplatin treatment.

Treatment	Hb (g/dl)	RBC (x 10^{12}/L)	WBC (x 10^{9}/L)
Control	13.45 ± 0.84	6.84 ± 0.58	6.85 ± 0.16
Cisplatin (24 h)	11.11 ± 0.12*	4.01 ± 0.20*	6.20 ± 0.19*
Cisplatin (48 h)	11.84 ± 0.44*	3.71 ± 0.59*	5.07 ± 0.20*
Cisplatin (72 h)	12.10 ± 0.76	4.15 ± 0.37*	3.21 ±0.17*
Cisplatin (96 h)	12.75 ± 0.60	5.88 ± 0.96	2.86 ± 0.19*
Cisplatin (120 h)	12.85 ± 0.51	5.67 ± 0.78	2.96 ± 0.16*

Control = Blood from untreated tumor-bearing mice;
Hb = Hemoglobin; RBC = Red blood cells;
WBC = White blood cells; Results are expressed as Mean ± S.D.
Student's t-test, n = 3-4, as compared to control, *$P \leq 0.05$.

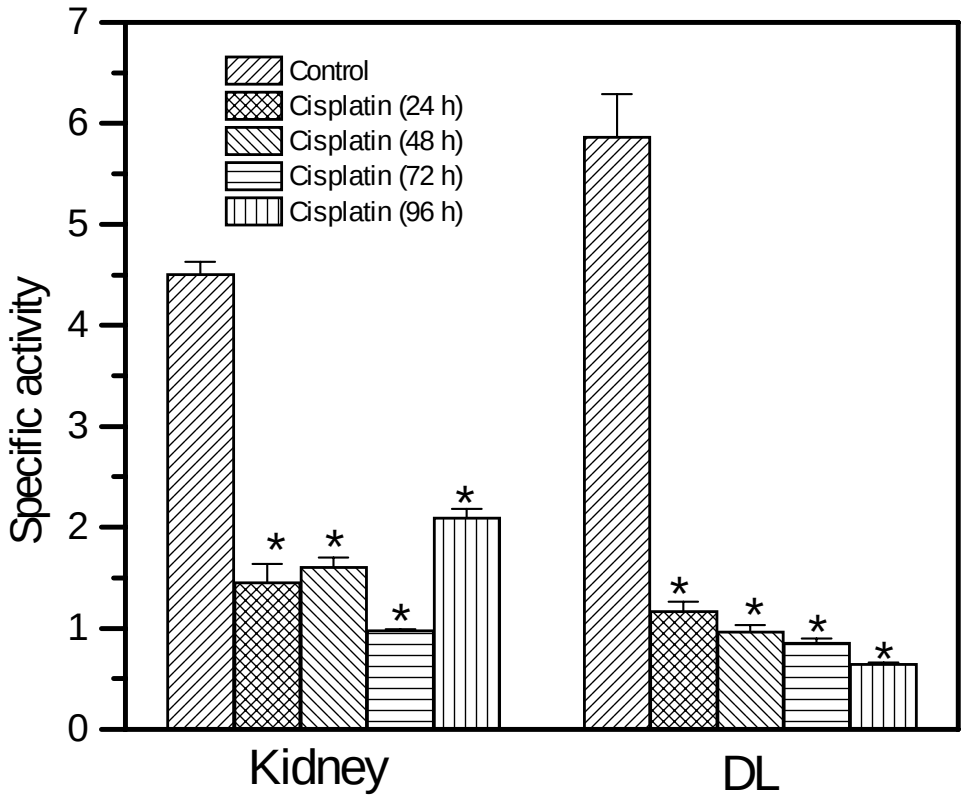

Fig. 4 : Changes in the specific activity of catalase in kidney and DL cells after cisplatin treatment. Student's t-test, n = 3-4, as compared to control, *P ≤0.05.

with 8 mg cisplatin) by the 5th day of treatment.

Cisplatin treatment of mice with subtherapeutic dose (4 mg/Kg body weight) caused a moderate tubular dilation and disorganized epithelial cells (Figure 1; C), while, the treatment with therapeutic dose (8 mg/Kg body weight) showed more tubular damage with the formation of atrophied tubules and less intact epithelial cells (Figure 1; D).

Cisplatin treatment of tumor-bearing mice caused damage in testes also indicating gonadal toxicity. The histological observation of testes from the control mice showed normal pattern of outer germinal epithelium, seminiferous tubules and interstitial cells in between the tubules (Figure 2; A, B). Here also, like that of kidney, increased damage and derangement to testicular cells were noticed with higher dose of cisplatin treatment as compared to lower dose. Single subtherapeutic dose (4 mg/Kg body wt.) of cisplatin caused a slight derangement in spermatogonial mass (Figure 2; C) while at therapeutic dose (8mg/Kg body wt.) vacuolated and broken seminiferous tubules were observed repeatedly (Figure 2; D). It has been

reported that cisplatin administration to rats induces significant hypoandrogenism, Sertoli cells dysfunction and lowered serum testosterone (Pogach *et al.*, 1981).

Since GSH, an important cellular antioxidant, is known to have protective functions in the cells against toxic effects of drugs and metals (Wang and Ballatori, 1998), the endogenous GSH levels were measured in the tissues. Cisplatin treatment of mice caused a significant reduction of GSH level in kidney (Table 1). A decrease of GSH in kidney after cisplatin treatment could lessen the protective mechanisms in the tissue thereby facilitating nephrotoxic effects. We have found that as compared to normal, non-tumorous animals, GSH level decreases significantly in the testes, but not in the kidney, of tumor-bearing animals (Khynriam and Prasad, 2003b). Cisplatin treatment in the present study has always been given to tumor-bearing mice, and, the decreased level of GSH in testes of tumor-bearing mice should also be helping to enhance cisplatin's toxic effects in testes. Thus, in both the tissues the involvement of GSH in developing toxic effects of cisplatin may be reflected. This suggestion is supported by the comparative histological changes observed in these tissues of mice treated with BSO, a known GSH depleting agent, plus cisplatin and respective dose of cisplatin alone. The combined BSO plus cisplatin treatment of mice caused enhanced tissue damage showing degenerated, damaged tubular lining membrane in kidney and broken seminiferous tubules in testes in a dose dependent manner (Figure 1, 2; E, F) as compared to that of respective dose of cisplatin alone (Figure1,2; D, C). It has been reported that depletion of GSH potentiates hepatotoxicity and nephrotoxicity caused by other drugs also (Kluwe and Hook, 1981; Kuo and Hook, 1982).

Cisplatin treatment of mice generates both superoxide and hydrogen peroxides increasing lipid peroxidation which are the main mediators of pathological changes at cellular levels (Aruoma, 1994). Cisplatin-mediated increase in mitochondrial lipid peroxidation in kidney has also been observed by Kharbangar *et al.*, 2000. At the same time it has also been known that GSH depletion may account for the enhanced lipid peroxidation (Nakano and Gemba, 1989) which may lead to tissue damage. Here also similar mechanism is proposed to be involved in developing tissue toxicity. GSH has also been reported to be essential in protecting germ cells and spermatozoa from cisplatin-induced genotoxicity[16] and oxidative injury (Malarvizhi and Mathur, 1996).

Determination of hematological parameters revealed that cisplatin treatment of mice caused a significant reduction in hemoglobin (Hb), RBC, WBC (Table 2) and blood GSH level (Table 1). The decreased level of Hb, RBC and WBC observed after cisplatin treatment may indicate the emergence of complications like myelosuppression and thrombocytopenia which are usually associated during cancer chemotherapy (Hoagland, 1982). Here, the development of these hematotoxicological

effects and concomitant decrease in blood GSH level may suggest that in blood also, like that of kidney or testes, changes in GSH level could be very critical in developing hematotoxicity. Low blood GSH has been reported during the condition of renal failure (Ross *et al.,* 1997). To understand clearly on this view, a selective GSH depleting agent (BSO), or cysteine a precursor for GSH synthesis (Ross *et al.,* 1997; Khynriam and Prasad, 2003) was also used (200mg/ Kg body wt.,) in combination with cisplatin treatment. It showed that as compared to that of cisplatin alone, all these hematological changes were further aggravated in BSO plus cisplatin treatment condition which should be because of greater decrease of blood GSH level (Figure 3). However, in cysteine plus cisplatin treated condition these hematotoxicological effects were very much reduced as compared to cisplatin alone or cisplatin plus BSO and it remained almost to level of untreated controls (Figure 3). This suggests that cisplatin-induced hematotoxicological effects are inversely related with the changes in blood GSH level and depict similar pattern as observed for kidney and testes.

Cisplatin treatment of tumor-bearing mice caused a significant decrease of GSH in DL cells initially (24-72 h), which was found to recover during later period (96 h) of treatment (Table 1). The reasons for this variation of GSH levels in DL cells are not clearly understood, but it does indicate that the cisplatin-mediated decrease in GSH is not permanent. However, it may be that the initial cisplatin-mediated decrease of GSH in tumor cells weakens their antioxidant defense and facilitates their killing but some surviving cells try to strengthen their antioxidant defense by recovering/taking more GSH later on. Cisplatin has also been known to have the interaction ability with GSH directly (Eastman, 1987) and the resulting GSH-platinum complexes could be eliminated actively from the cells (Ishikawa and Ali-Osman, 1993). Here also an initial decrease in cellular GSH should be enhancing the cellular uptake and subsequent increased binding of drug to the DNA leading to cytotoxic effect. Any damage exerted on cellular DNA at the early stage of treatment seems to be very critical in resulting the antitumor effect. A decrease in the rate of cancer cell proliferation has also been correlated with a decrease in GSH in tumor cells (Estrela *et al.,* 1992).

In an attempt to further understand on the significance of GSH in the cisplatin-mediated toxicological and cytotoxicity effects, the activity of catalase was also assayed in kidney and DL cells. Cisplatin treatment induced GSH depletion may account for the enhanced lipid peroxidation (Nakano and Gemba, 1989). Catalase catalyzes decomposition of H_2O_2 to H_2O and O_2 and thus protects cells from oxidative damage (Ueda *et al.,* 1990). The results from catalase assay showed that the enzyme activity was severely reduced (~ 60-70%) after cisplatin treatment (Figure 4). This cisplatin-mediated inhibition of catalase activity should also be helping to increase the lipid peroxidation, which is partially achieved by decrease in GSH.

Thus, these various findings suggest that during cisplatin chemotherapy decreased GSH level, thus, lowering antioxidant state of the tissues as well as DL cells could play a critical role in developing cisplatin mediated-tissue toxicity as well as DL cytotoxicity leading to antitumor effect in the hosts.

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