Dietary Ascorbic Acid-Mediated Augmentation of Antitumor Activity and Protection Against Toxicities Induced by *Cis*-Diamminedichloroplatinum-(II) in Dalton's Lymphoma-Bearing Mice

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Abstract: *Cis*-Diamminedichloroplatinum-(II) (CDDP) commonly known as cisplatin is considered as a major anticancer drug against a broad spectrum of malignancies. This study evaluates the modulatory effect of dietary ascorbic acid (AA) on the therapeutic efficacy of CDDP against murine ascites Dalton's lymphoma (DL) and some tissue toxicities in tumor-bearing mice.

As compared to CDDP alone, the combination treatment with ascorbic acid (AA) plus CDDP showed better therapeutic efficacy against murine ascites Dalton's lymphoma. DL cells treated with CDDP showed the appearance of apoptotic features involving fragmentation of nucleus into discrete masses and plasma membrane blebbing. As compared to CDDP alone, combination treatment caused an increase in the number of apoptotic DL cells. Reduced glutathione (GSH) level was noted to decrease in DL cells while it increased in kidney after combination treatment. Blood haemoglobin (Hb), red blood cells (RBCs) and white blood cells (eosinophils, basophils and lymphocytes) were also decreased after CDDP treatment while overall betterment in hematological parameters was noted after combination treatment. The analysis of renal function tests (RFT) and liver function tests (LFT) suggest an improvement against CDDP-induced liver and kidney toxicities after combination treatment.

The decrease in GSH levels particularly in DL cells and an increase in kidney and liver after combination treatment may have a role in the antitumor activity and decrease in CDDP-induced toxicity in the tumor-bearing host. Improvement in the LFT, RFT and hematological toxicities after combination treatment may have a beneficial effect in the improved survival of tumor-bearing mice.

Keywords: Ascorbic acid, Apoptosis, *cis*-Diamminedichloroplatinum-(II), Dalton's lymphoma, Reduced glutathione, Hematotoxicity.

1. INTRODUCTION

Cancer and its treatment are among the most critical health issues. According to world cancer report released by the World Health Organization (WHO) in 2003, cancer rates could further increase by 50% to 15 million new cases in the year 2020 [1]. Chemotherapy has proved to be an effective treatment regime which can be used either singly as monotherapy or in combination with surgery and/or radiotherapy against various types of cancers.

Cis-diamminedichloroplatinum-(II) or CDDP is a platinum-containing inorganic, square-planar complex which is well-known anticancer agent being used against a wide spectrum of malignancies including testicular, head and neck, ovarian, cervical, non-small cell lung carcinoma, and many other types of cancer [2, 3]. The ability of CDDP to react with DNA and formation of CDDP-DNA adducts with inter- and intra-strand nuclear DNA crosslinks is suggested to be the main mechanism underlying its cytotoxic effect [4]. It is noted that 1, 2- intrastrand ApG and GpG are the major forms of the DNA adducts, accounting for about 85-90% of total adducts [5]. In addition to its interaction with cellular DNA, the changes in various biochemical/enzymatic parameters, immune response, cell surface structure have also been observed which have led to propose the involvement of multistep and multilevel effects of CDDP in the tumor cells/host [6]. However, the efficacy of CDDP is often hampered by the development of various side effects such as nephrotoxicity, myelosuppression, neurotoxicity, ototoxicity in the host [7] and acquired resistance by cancer cells [8]. Hence, in an attempt to overcome these impediments in the host, the uses of CDDP in combination with some modulating agents [9-10] have been tried with different degrees of success. In an endeavour to decrease drug-induced toxicity in the host without decreasing the therapeutic efficacy, the use of anticancer drugs such as cyclophosphamide [11], paclitaxel [12] and arsenic trioxide [13] in combination with ascorbic acid (vitamin C) have also been examined.

Ascorbic acid (L, 3-ketothreohexuronic acid lactone) or vitamin C is a water soluble vitamin with antioxidant properties. It is an active reducing agent involved in

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various biological effects and plays an important role in the metabolism and detoxification of many endogenous and exogenous compounds [14]. Vitamin C has a long history of adjunctive use in cancer therapy but the definite use of vitamin C for the treatment of cancer still remains inconclusive [15]. While many studies have reported good therapeutic potential of vitamin C against cancer [16-18], some have shown virtually no benefit from vitamin C treatment [19]. Role of ascorbic acid has also been suggested in inhibiting carcinogenesis [20-22] or enhancing carcinogenesis [23, 24]. Some genotoxic effects of vitamin C *in vitro* test systems has been demonstrated [25, 26] but in the experiments *in vivo*, there are no genotoxic effects by vitamin C. Vitamin C has been reported to increase the antineoplastic activity of doxorubicin, CDDP and paclitaxel against human breast carcinoma cells [27].

Most of the studies involving modulatory effects of ascorbic acid on the antitumor effects or toxicities induced by various anticancer agents have been done separately in the cancer cell lines/tumor-bearing hosts or in normal animals respectively. It is reasonable that the ascorbic acid-mediated protective strategies should be tested in tumor-bearing animals, because prospective protective agents may also affect the anti-tumor activity of the anticancer drugs and also because tumors alter the metabolic and endocrine equilibrium in the host [28].

Thus, considering the variable findings on the significance of vitamin C in relation to cancer chemotherapy and possibility of development of CDDP-induced side effects, the novelty of the present study lies to assess the modulatory effect of dietary ascorbic acid on CDDP mediated antitumor efficacy and toxicity in the same host with experimental malignant murine tumor and it has the significance in cancer treatment in general. The basic findings of the work reveal that combination treatment *in vivo* with ascorbic acid plus CDDP has better therapeutic efficacy than CDDP alone against Dalton's lymphoma and it involves an increase in the number of apoptotic cells and a decrease in GSH in DL cells. At the same time, combination treatment resulted in an improvement against CDDP-induced toxicities in the host.

2. MATERIALS AND METHODS

2.1. Chemicals

Reduced glutathione, 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, Mo, USA. Cisplatin solution (1 mg/ml of 0.9%, NaCl) was obtained from Biochem Pharmaceutical Industries, Mumbai, India. L-ascorbic acid (vitamin C) was purchased from HiMedia laboratories, Mumbai, India. Ethylenediaminetetra-acetic acid (EDTA) and other chemicals used in the experiments were of analytical grade and purchased from SRL Pvt. Ltd., Mumbai, India.

2.2. Animals and Tumor Maintenance

Mice colony was maintained under conventional laboratory conditions at room temperature of 22 ± 2°C keeping 5-6 animals per propylene cage using paddy husk as bed with food pellets (Amrut Laboratory animal feeds, New Delhi) and drinking water *ad libitum*. Inbred Swiss albino male mice in the age group of about 10-12 weeks weighing about 25-28g were used for the experiments.

The malignant tumor, Dalton's lymphoma, was originated in the thymus gland of DBA/2 mouse at the National Cancer Institute, Bethesda, MD, USA in 1947 and subsequently an ascites form was developed by repeated intraperitoneal (i.p.) transplantation of the tumor [29]. Later, in India probably this tumor cell line was first procured by cancer Research Institute, Mumbai. Ascites Dalton's lymphoma (DL) is being maintained *in vivo* in mice of both sexes by serial intraperitoneal (i.p.) transplantations of approximately $1x10^7$ viable tumor cells per animal (0.25 ml in phosphate-buffered saline (PBS), pH 7.4). Tumor transplanted hosts usually survived for 19-21 days.

The maintenance, use of these animals and the experimental protocol of the present study was approved by the institutional ethical committee, North-Eastern Hill University, Shillong.

2.3. Drug Treatment Schedule

Based on earlier reports [2, 6, 30], the therapeutic dose of CDDP was selected as 10mg/kg body weight. Similarly, the dose of ascorbic acid was selected to be 1% in drinking water which has already been standardized as an effective dose [11, 31]. The day of tumor transplantation was taken as day '0'. Tumor transplanted mice were randomly divided into four groups consisting of 10 mice in each group. Group-I mice served as tumor-bearing control and received normal saline (0.9%, NaCl) only. Group-II mice were given 1% ascorbic acid (17.65-19.20 mg/day/per

animal) through drinking water for 5 consecutive days starting from the 5th day post-tumor transplantation. Group-III mice were injected intraperitonially with a single dose of CDDP (10 mg/kg body weight) on the 10th day post-tumor transplantation. Group-IV mice received ascorbic acid through drinking water from the 5th day post-tumor transplantation and were administered with CDDP (i.p., 10 mg/kg body weight) on the 10th day of tumor growth. Similarly for the estimation of GSH and protein in DL, liver and kidney, total 10 mice were kept in each group and two animals were sacrificed by cervical dislocation on 1st, 2nd, 3rd and 4th day after CDDP treatment and tissues were collected separately. The experiments were repeated three times.

2.4. Hosts Survival and Antitumor Study

The survival patterns of animals in each group were monitored daily and deaths, if any, were recorded. The antitumor efficacy was expressed in percentage of average increase in life span (% ILS), and was calculated using the formula: (T/C x 100)-100, where, T and C are the mean survival days of treated and control group of mice respectively. The average body weight of mice in different groups was also recorded daily. The change in body weights of mice in different treatment groups may indirectly indicate the changes in tumor growth/volume.

2.5. Apoptosis Study Using Fluorescence Microscopy

Fluorescence-based determination of apoptosis in DL cells was done using acridine orange and ethidium bromide (AO/EtBr) staining method as described by Baskic *et al*. [32] and also used earlier in our laboratory [33]. After treatment of tumor-bearing mice with AA, CDDP, AA plus CDDP for 24, 48, 72 and 96 h, DL cells were collected, washed with PBS and treated with AO/EtBr (100 µg/ml PBS of each dye). The cells were thoroughly examined under a fluorescence microscope (Leica), photographed and compared with that of control. Viable cells' nuclei stain green due to permeability of only acridine orange whereas, apoptotic cells appear red/orange due to co-staining of both the fluorescent dyes. Based on the score of apoptotic and viable cells under the microscope, apoptotic index was determined.

2.6. Scanning Electron Microscopy

Dalton's lymphoma ascites collected from the peritoneal cavity of mice at different time points were centrifuged at 1000xg for 10 min at 4°C. The cells were washed in PBS (pH 7.4) and cell pellets were resuspended in PBS (1:4, w/v) and fixed in 2.5% (v/v) glutaraldehyde at 4°C. Fixed cells were rinsed in phosphate buffer (0.1 M, pH 7.4) and post-fixed with 1% osmium tetroxide. Cells were rinsed with PBS and dehydrated with an ascending grade of acetone (30, 50, 70, 90, and 100 % for 10 min each). The cells were then dried by critical point-drying method substituting dry acetone from the cells by CO_2 in a critical point dryer (CPD-030, BAL-TEC Co.). The dried cells were affixed to an aluminium stub with double-stick tape, coated with gold in a fine coat ionic sputter (SCD-005, BAL-TEC Co.). The cells were thoroughly viewed and photographed under scanning electron microscope (JEOL JSM – 6360).

2.7. Reduced Glutathione (GSH) Estimation

Total reduced glutathione (GSH) in liver, kidney and DL cells was determined using the method of Sedlak and Lindsay [34]. Briefly, 5% tissue homogenates of DL cells, kidney or liver were prepared in 0.02 M EDTA (pH 4.7). Total GSH was determined by adding the tissue homogenate or pure reduced form of glutathione (100 µl) to 0.9 ml of 0.02 mol/L EDTA, pH 4.7 and 1 ml of 0.2 mol/L Tris–EDTA buffer, pH 8.2, and followed by 20 µl of Ellman's reagent (10 mmol/L DTNB in methanol). After 30 min of incubation at room temperature, the reaction mixture was centrifuged at 3000xg and the absorbance of the clear supernatant was read against a reagent blank at 412 nm in a Varian Carey-50 spectrophotometer. The results were read from a standard curve prepared from 1mmol/L solution of reduced glutathione.

2.8. Protein Estimation

Protein content in the liver, kidney and DL cells was determined following the method of Lowry *et al.* [35] using bovine serum albumin (BSA) as a standard.

2.9. Hematological Studies

Red blood cells (RBC) counts, white blood cells (WBC) counts and haemoglobin estimation was carried out according to the method described by Dacie and Lewis [36]. The blood for hematological studies was collected from the tail veins without killing the mice. For differential leukocyte counts (DLC), a drop of fresh blood from the mice in different groups was taken on clean slides and a thin and uniform blood film was prepared with the help of another clean slide. The

Table 1: Antitumor Activity of AA and CDDP Used Alone and in Combination Against Murine Ascites Dalton's Lymphoma

Treatment groups	Day of treatment	Route of treatment	Survival days (Mean ± S.D.)	ILS (%)
Group- I (Control)	-	-	19.2 ± 1.9	-
Group- II	5th day onwards (AA)	oral	34.5 ± 3.0*	79.68
Group- III	10th day (CDDP)	i.p.	42.7 ± 3.4*	122.39
Group IV	5th day onwards (AA) 10th day (CDDP)	oral i.p.	46.6 ± 3.7*, #	142.71

Values represent the mean ± S.D., n =3, Student's t-test, *$P \leq 0.05$ as compared to control; #$P \leq 0.05$ as compared to CDDP. Control = Tumor bearing mice without AA or CDDP treatment; AA= ascorbic acid; CDDP = *cis*-diamminedichloroplatinum-(II), %ILS = % increase in life span.

blood film was air dried for overnight, stained with Leishman's stain the following day and mounted in DPX. Counting was done under microscope in a narrow longitudinal strip of the blood film starting from one end of the film to the other end. The number of different types of white blood cells (neutrophils, basophils, monocytes, lymphocytes and eosinophils) were recorded and expressed in percentage.

2.10. Liver and Renal Function Tests

For biochemical analysis of liver function test (LFT) and renal function test (RFT) mice in different groups were sacrificed on the 4th day of CDDP treatment. Blood was collected directly from the heart and serum was separated, stored at -180°C until the analysis could be completed. Biochemical analyses included measurement of the activities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) along with urea and creatinine level. The measurements of these biochemical parameters were done in Clinical Chemistry Analyzer (SYNERGY BIO-1904C) at North-east Diagnostic centre, Shillong.

2.11. Statistical Analysis

The significance of difference in two experimental groups was statistically tested using Student's t-test and $P \leq 0.05$ was considered significant.

3. RESULTS

3.1. Host Survival Patterns

Following tumor transplantation, the increase in belly size and body weight, with sluggish movement of the animal was noted from 3-4th day onwards depicting an early sign of tumor development. Control tumor-transplanted mice survived for about 19-20 days. The group of mice treated with AA [Group-II] or CDDP alone [Group-III] showed the ILS of about 79% and 122% respectively. Further increase in the survival time (ILS ~ 142%) was observed in mice under combination treatment of AA plus CDDP (Table **1**).

A regular increase in body weight of mice was noted in control group which may indicate the tumor growth and increase in tumor volume. CDDP treatment of tumor bearing mice or AA + CDDP treatment caused a decrease in body weight which may be correlated with decrease in tumor volume as compared to control. AA alone treatment also showed decrease in body weight (Figure **1**).

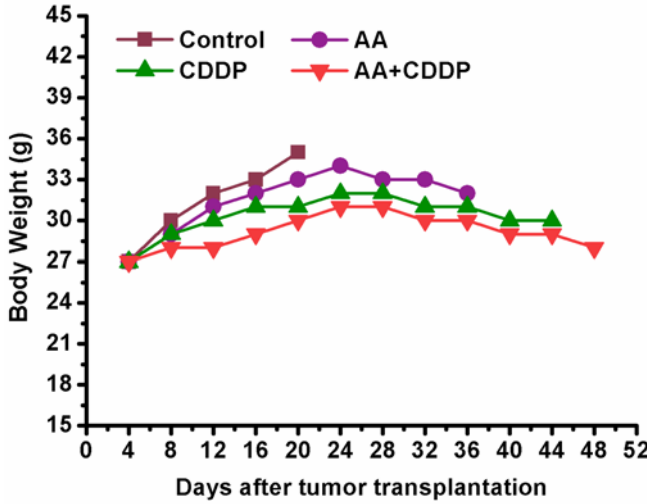

Figure 1: Graphs showing the changes in the average body weight (g) of tumor-bearing mice in control and different treated groups.

3.2. Apoptosis Study

As illustrated in Figure **2**, light green nuclei (viable cells) with normal membrane structure were seen in the control group (Figure **2a**). Different apoptotic features

were observed in DL cells at different time intervals after combination treatment and these include membrane blebbing, nuclear condensation (Figure **2b**) and fragmentation (Figure **2c-e**), membrane disintegration and appearance of cytoplasmic vacuoles (Figure **2c**, **d**). The time dependent increase in apoptotic features were observed in DL cells from all treated groups (AA, CDDP and AA plus CDDP) and only the combined treated DL cells' photographs are shown in Figure **2**. Determination of apoptotic index revealed that as compared to CDDP alone treatment, number of apoptotic cells significantly ($P \leq 0.05$) increased in AA plus CDDP treated group during 24 to 96 h of treatment (Figure **3**). AA alone treatment also caused some increase in the number of apoptotic cells in time dependent manner (Figure **3**).

3.3. Scanning Electron Microscopic Study

Scanning electron microscopy of the DL cells from control group showed almost rounded shape with few membrane projections and ruffles distributed evenly over the cell surface (Figures **4a** & **5a**). CDDP treatment caused a reduction in ruffles/microvilli (Figure **4b-e**) and appearance of membrane blebs (Figure **4c**), membrane fusion (Figure **4c-e**) and plasma membrane deformities (Figure **4d**, **e**). Combination treatment of mice with AA plus CDDP also revealed more or less similar pattern of deformities in DL cells morphology which include appearance of membrane blebs (Figure **5c**), loss in microvilli from the cell surface (Figure **5d**, **e**) and cell membranes folding and shrinkage (Figure **5d**, **e**).

3.4. Reduced Glutathione (GSH) Levels

GSH levels in DL cells increased with tumor growth, reaching maximum on the day 10, thereafter, it decreased slightly over the next 4-5 days (Figure **6**). The changes in GSH level in liver, kidney and DL cells under different treatment conditions are shown in

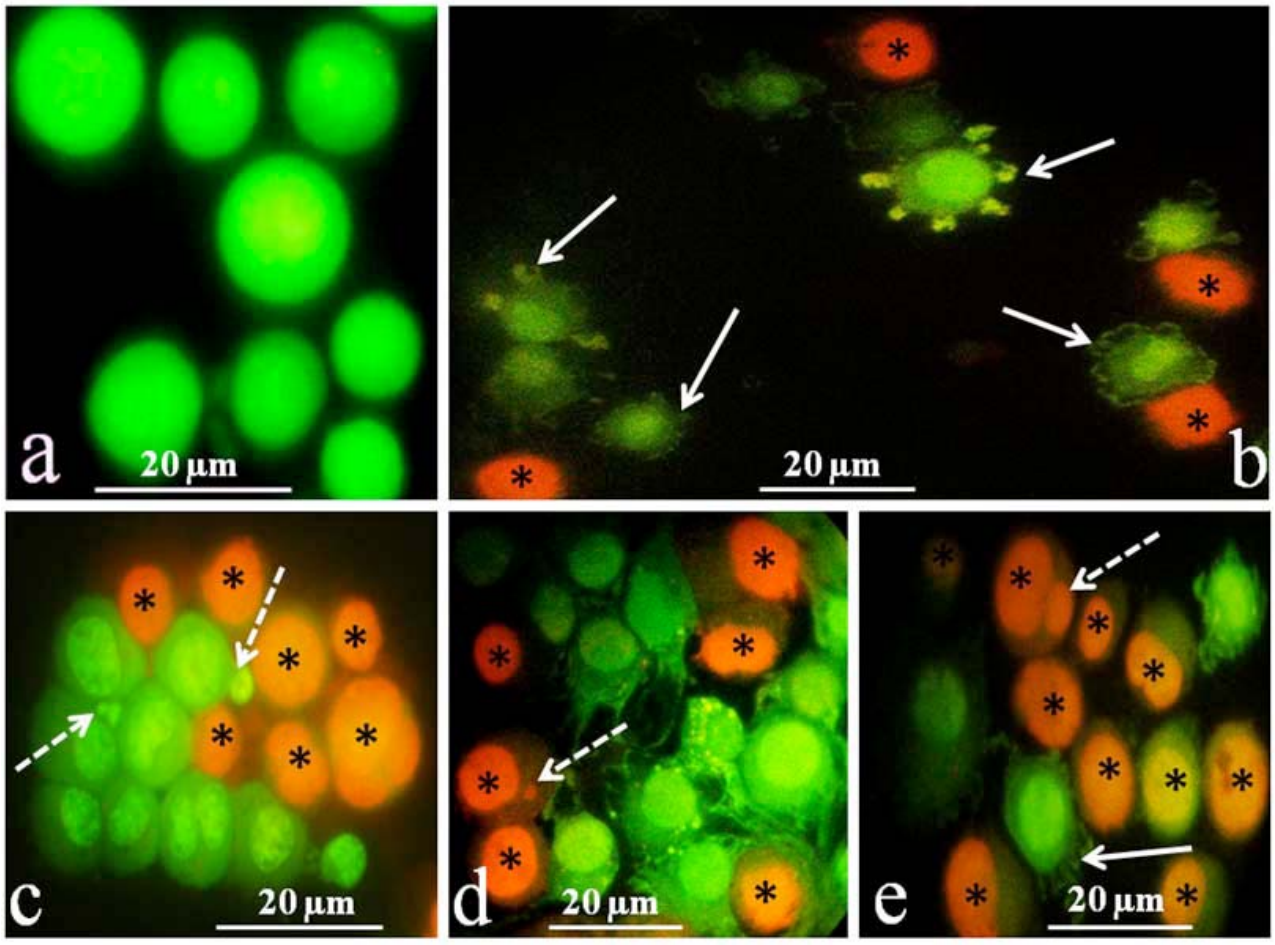

Figure 2: Acridine orange-ethidium bromide staining of Dalton' lymphoma (DL) cells under different treatment conditions *in vivo*. Control DL cells (**a**); Combination treatment with AA plus CDDP at- 24 h (**b**), 48 h (**c**), 72 h (**d**) and 96 h (**e**). Each experiment was performed in triplicate which showed similar morphological features. Dotted arrow indicates fragmented nuclei, regular arrow showing membrane blebbing and asterisk represents apoptotic cells.

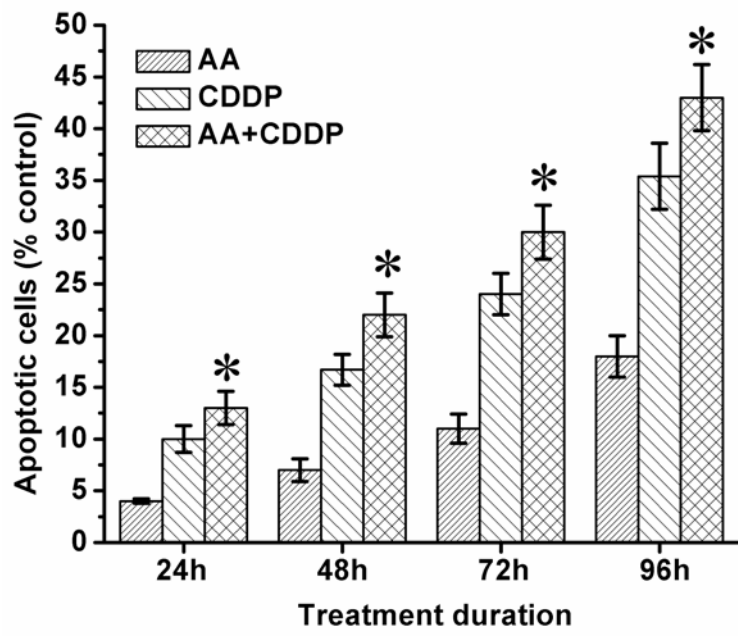

Figure 3: Apoptotic index (percent apoptotic cells) of Dalton's lymphoma (DL) cells from the mice treated with AA, CDDP and AA plus CDDP. The result is based on the analysis of live and apoptotic cells following acridine orange-ethidium bromide (AO/EtBr) staining. Thousand cells were analyzed and percentage of apoptotic cells was calculated. Results are expressed as mean ± S.D. Student's t-test, n = 5, *P ≤ 0.05 as compared to CDDP treatment alone.

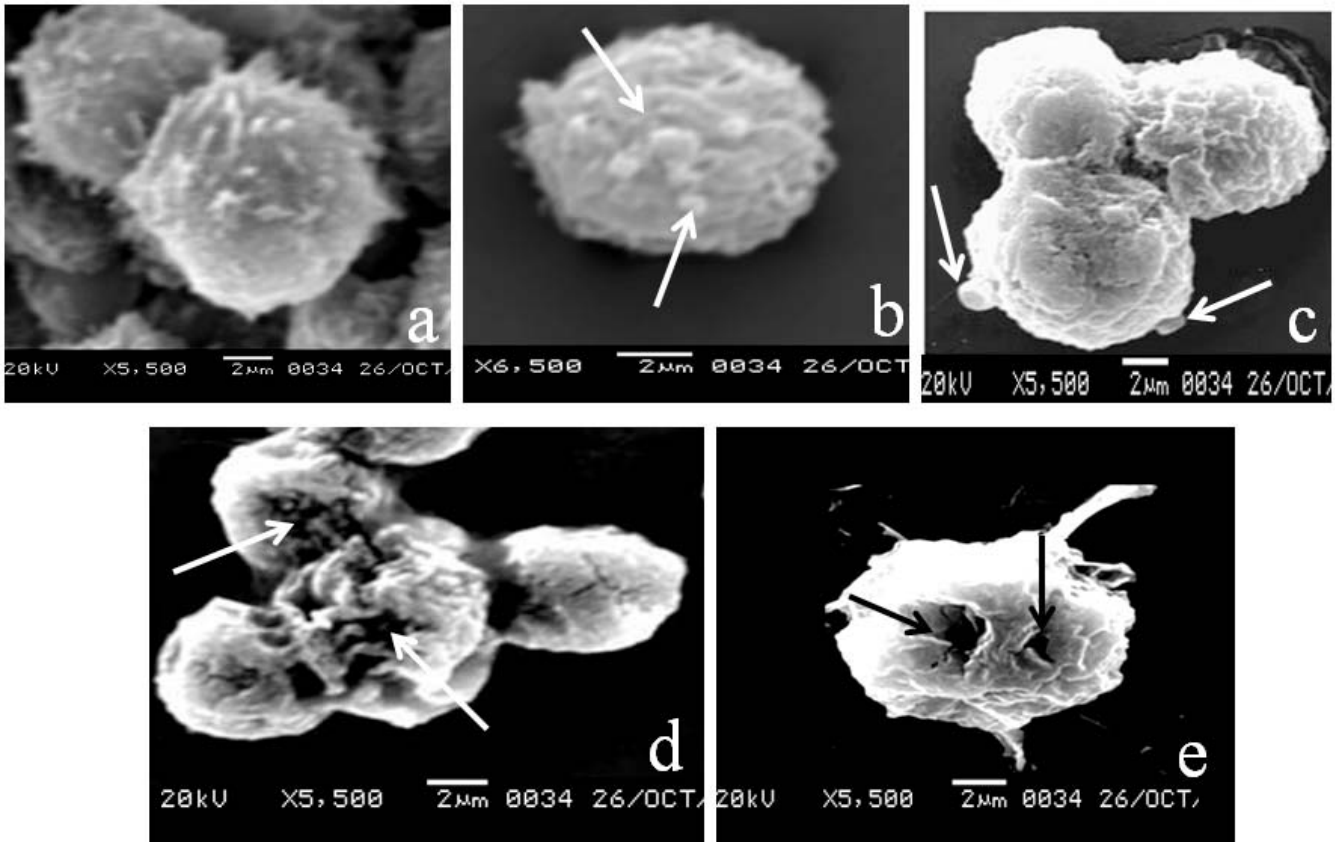

Figure 4: Scanning electron micrographs of Dalton's lymphoma (DL) cells. Control DL cells (**a**), and DL cells after CDDP treatment for 24 h (**b**), 48 h (**c**), 72 h (**d**) and 96 h (**e**). Control DL cells showed rounded shape with few membrane projections and ruffles distributed evenly over the cell surface. Arrows indicate important cellular features on the cells such as microvilli (**b**), membrane blebs (**c**), cell membrane fusion (**c**, **d**) and cell membrane deformities (**d**, **e**) noted after CDDP treatment.

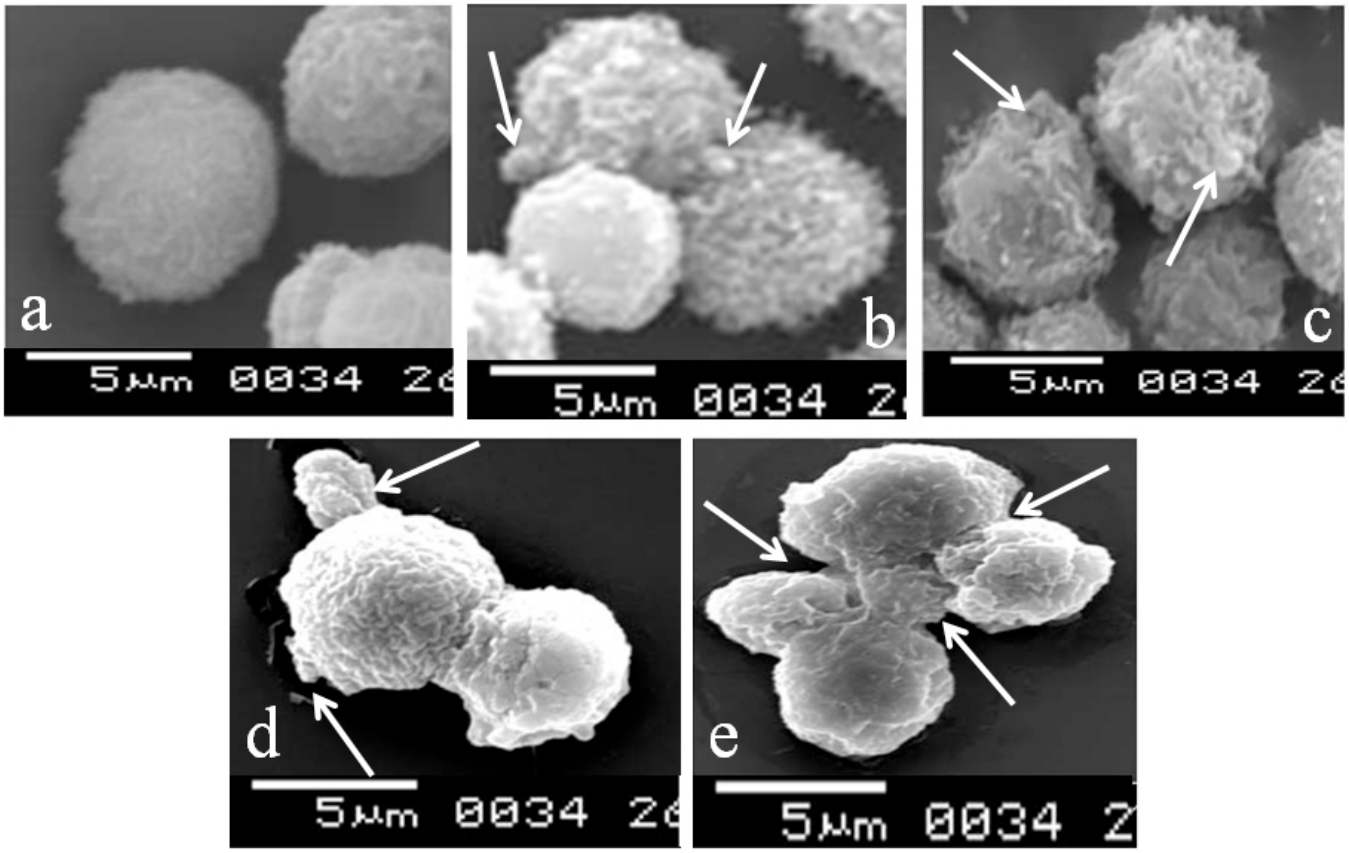

Figure 5: Scanning electron micrograph of DL cells. Control DL cells (**a**), and DL cells after AA plus CDDP treatment for 24 h (**b**), 48 h (**c**), 72 h (**d**) and 96 h (**e**). Arrows indicate important cellular features on the cells such as microvilli (**b**), membrane ruffles and blebs (**c**), membrane fusion (**d**) and cell membrane deformities (**d**, **e**).

Figure **7**. The level of GSH was found to be higher in liver followed by kidney and DL cells. The CDDP treatment resulted in a significant decrease in GSH level in liver (Figure **7a**). The level of GSH in liver was restored to approximately control levels at 72-96 h of treatment with AA plus CDDP. Similar pattern was also observed in the kidney also (Figure **7b**).

CDDP treatment (24-96 h) or combination treatment with AA plus CDDP of tumor-bearing mice caused a significant decrease in GSH level in DL cells (Figure **7c**). Combination treatment showed lowest GSH level in DL cells. As compared to CDDP alone, there was no significant difference in GSH level in DL cells after AA plus CDDP treatment.

3.5. Protein Content

CDDP treatment of mice caused a decrease in protein levels in liver, kidney and DL cells (Figure **8**). A recovery in protein content was noted in liver during 72-96 h of combine treatment (Figure **8a**). AA plus CDDP treatment in liver and kidney showed little improvement in protein level from 24-96 h of treatment (Figure **8**).

3.6. Hematological Studies

As compared to tumor-bearing control, CDDP treatment caused a decrease in the haemoglobin

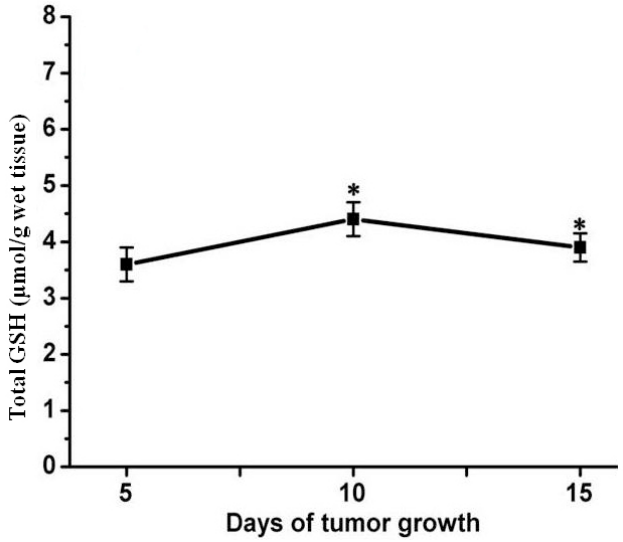

Figure 6: Changes in total reduced glutathione (GSH) content in the DL cells at different stages of tumor growth. Results are expressed as mean ±S.D. Student's t-test, n=3. *P ≤ 0.05, as compared to the 5th day of tumor growth.

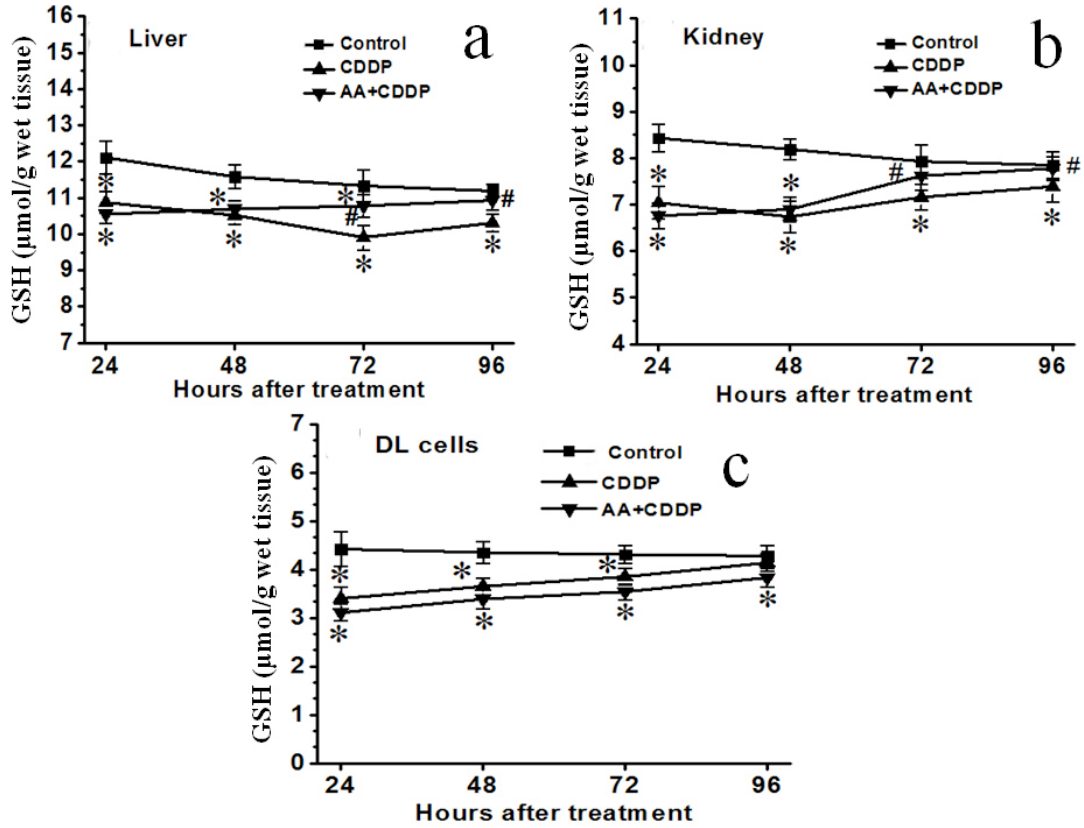

Figure 7: Changes in total reduced glutathione (GSH) content in the liver (**a**), kidney (**b**) and DL cells (**c**) at different treatment conditions. Results are expressed as mean ± S.D. Student's t-test, n=3, *P ≤ 0.05, as compared to respective corresponding control and #P≤ 0.05, as compared to respective CDDP treatment.

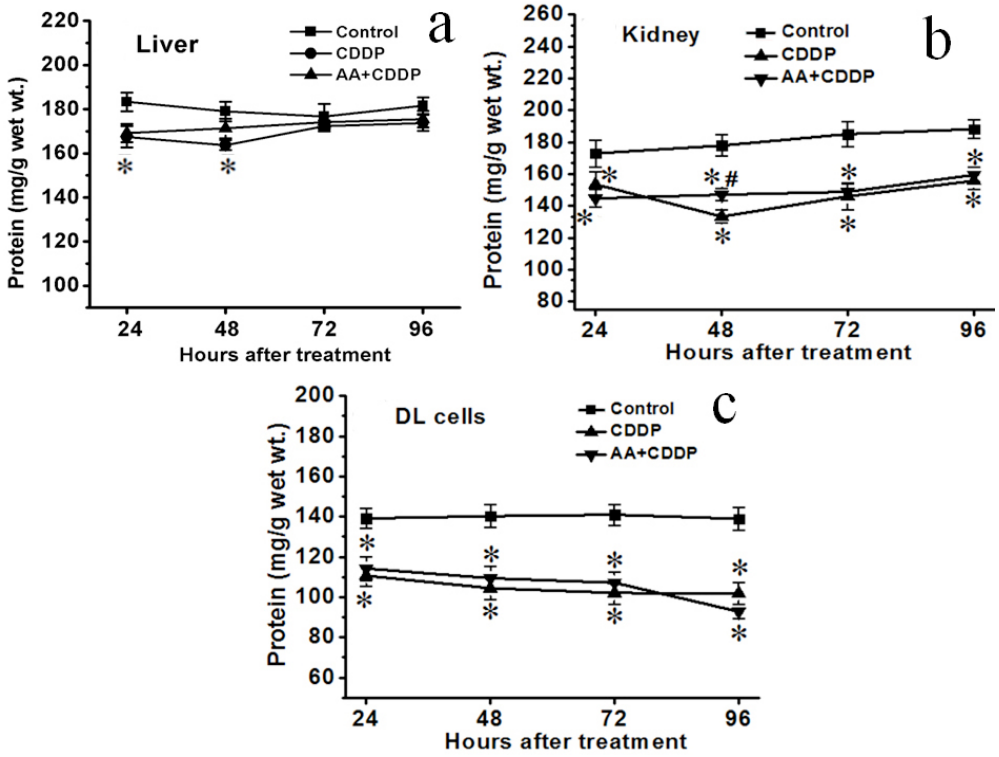

Figure 8: Changes in protein content in liver (**a**), kidney (**b**) and DL cells (**c**) of tumor-bearing mice at different treatment conditions. Results are expressed as mean ± S.D. Student's t-test, n=3, *P ≤ 0.05, as compared to respective corresponding control.

Table 2: Changes in the Hematological Values of Normal and Tumor-Bearing Mice Under Different Treatment Conditions

Treatment groups	RBC(x10^{12}/l)	WBC(x10^{9}/l)	Hb (g/dl)
Normal-	7.16 ± 0.44	04.53 ± 0.35	14.51 ± 0.86
Control- 24 h	6.83 ± 0.39	09.51 ± 0.34	11.82 ± 0.58
48 h	6.29 ± 0.88	10.01 ± 0.29	11.39 ± 0.52
72 h	6.20 ± 0.57	10.26 ± 0.46	10.74 ± 0.71
96 h	5.91 ± 0.10	10.57 ± 0.35	09.91 ± 0.45
CDDP treated- 24 h	4.65 ± 0.35*	3.22 ± 0.30*	8.13 ± 0.54*
48 h	3.79 ± 0.57*	4.27 ± 0.18*	8.15 ± 0.94*
72 h	4.42 ± 0.21*	5.04 ± 0.72*	8.49 ± 0.60*
96 h	5.74 ± 0.70	5.44 ± 0.43*	7.01 ± 0.42*
AA+CDDP treated- 24 h	5.74 ± 0.29$^{\#}$	4.09 ± 0.19$^{\#}$	11.59 ± 0.52$^{\#}$
48 h	6.29 ± 0.52$^{\#}$	4.69 ± 0.21$^{\#}$	10.79 ± 0.23$^{\#}$
72 h	5.86 ± 0.30$^{\#}$	5.53 ± 0.33$^{\#}$	09.91 ± 0.33$^{\#}$
96 h	5.68 ± 0.32	6.13 ± 0.50$^{\#}$	10.56 ± 0.91$^{\#}$

Values represent mean ± S.D. Student's t-test, n = 3, , *$P \leq$ 0.05, as compared to corresponding control; $^{\#}P \leq 0.05$ as compared to CDDP. Normal = hosts without tumor or any treatment condition; Control = Untreated tumor-bearing hosts receiving drug vehicle normal saline alone; AA = Ascorbic acid; CDDP = *cis*-diamminedichloroplatinum-(II), RBC = Red blood cells, WBC = White blood cells, Hb = Haemoglobin.

content, RBC and WBC counts. As compared to CDDP alone, combination treatment resulted in significant recovery in haemoglobin concentration, RBC and WBC counts (Table **2**). As compared to control, CDDP treatment of tumor-bearing mice caused a significant decrease in lymphocytes, eosinophils and basophils while an increase was observed in monocytes and neutrophils (Table **3**). Combination treatment caused an increase in lymphocytes, eosinophils and a decrease in monocytes, neutrophils and basophils (Table **3**).

3.7. Analysis of Renal and Liver Function Test

Serum urea and creatinine levels were studied to monitor the changes in renal toxicity. As compared to that of control DL-bearing mice, serum urea and creatinine levels were significantly elevated in CDDP-treated mice. The increase in serum urea and creatinine level was ~270% and ~302% respectively. However, as compared to CDDP alone, combination treatment of animals with AA plus CDDP significantly brought down the elevated levels of serum urea and

Table 3: Differential WBC Counts in the Blood of Normal and Tumor-Bearing Mice Under Different Treatment Conditions

Treatment groups	Lymphocytes	Monocytes	Eosinophils	Neutrophils	Basophils
Normal -	65.69 ± 4.24	3.89 ± 0.42	1.61 ± 0.12	27.63 ± 2.56	1.10 ± 0.12
Control - 24h	28.22 ± 4.98	2.11 ± 0.45	5.23 ± 0.42	64.52 ± 3.19	1.19 ± 0.15
48h	26.80 ± 2.43	2.07 ± 0.25	5.34 ± 0.21	67.14 ± 2.67	1.15 ± 0.26
72h	24.46 ± 2.29	1.97 ± 0.37	5.69 ± 0.33	69.04 ± 2.05	1.08 ± 0.18
96h	21.63 ± 3.16	1.92 ± 0.28	4.90 ± 0.20	70.49 ± 2.73	1.06 ± 0.15
CDDP treated- 24h	18.13±3.06*	2.46 ± 0.38*	4.92 ± 0.36*	72.17 ± 2.58*	0.79 ± 0.13*
48h	23.57±3.14*	2.64 ± 0.31*	4.39 ± 0.19*	69.89 ± 3.45*	0.67 ± 0.10*
72h	22.45±2.90*	2.32 ± 0.25*	5.17 ± 0.32	67.51 ± 3.11*	0.53 ± 0.12*
96h	20.18±3.32	2.31 ± 0.28*	5.53 ± 0.27*	70.92 ± 2.89	0.85 ± 0.16*
AA+CDDP treated- 24h	28.92 ± 2.53$^{\#}$	2.39 ± 0.23$^{\#}$	5.51 ± 0.34$^{\#}$	63.01 ± 3.16$^{\#}$	0.62 ± 0.10$^{\#}$
48h	36.01 ± 1.88$^{\#}$	2.15 ± 0.41$^{\#}$	5.46 ± 0.27$^{\#}$	55.11 ± 2.56$^{\#}$	0.59 ± 0.14$^{\#}$
72h	31.38 ± 2.17$^{\#}$	2.20 ± 0.29$^{\#}$	5.89 ± 0.30$^{\#}$	59.77 ± 2.12$^{\#}$	0.65 ± 0.13$^{\#}$
96h	28.64 ± 2.01$^{\#}$	2.25 ± 0.31	5.72 ± 0.22	62.13 ± 2.61$^{\#}$	0.74 ± 0.11$^{\#}$

Values represent % mean ± S.D. Student's t-test, n = 3, Student's t-test, *$P \leq$ 0.05, as compared to the corresponding control; $^{\#}P \leq$ 0.05 as compared to CDDP. Normal = Mice without tumor or any treatment condition; Control = Untreated tumor-bearing mice receiving drug vehicle normal saline alone; AA = Ascorbic acid; CDDP= *cis*-diamminedichloroplatinum-(II), DL = Dalton's lymphoma**.**

Table 4: Serum Urea and Creatinine Level in Normal and Tumor-Bearing Mice Under Different Treatment Conditions

Treatment groups	Urea (mg/dl)	% control	Creatinine (mg/dl)	% control
Normal	52.0± 4.0	-	0.49±0.04	-
Control	73.0±4.2	-	0.72±0.12	-
AA	66.6±3.2*	-8.76	0.54±0.11*	-25
CDDP	270.6±3.7*	270.68	2.9±0.14*	302.78
AA+ CDDP	122.6±3.2*, #	67.95	1.2±0.11*, #	66.67

Mice were sacrificed on the 4th day of CDDP treatment. Values are mean ± S.D., n =3, Student's t-test. *$P \leq 0.05$ as compared to respective control; #$P \leq 0.05$ as compared to CDDP. Normal = mice without tumor or any treatment condition; Control = Untreated tumor-bearing mice receiving drug vehicle normal saline; AA = Ascorbic acid; CDDP=*cis*-diamminedichloroplatinum-(II). DL = Dalton's lymphoma.

creatinine, indicating a protective role of AA (Table **4**). ALT, AST and ALP activity were studied to assess hepatotoxicity in the hosts under different treatment conditions. An increase in ALT, AST and ALP activity was observed after CDDP treatment, which was decreased after combination treatment (Table **5**).

4. DISCUSSION

In cancer chemotherapy, CDDP has been frequently used in combination with one or more anticancer drugs, with better results [37]. The findings from the present study showed that ascorbic acid plus CDDP combination treatment has better therapeutic efficacy against murine ascites Dalton's lymphoma. The tumor bearing mice treated with CDDP alone depicted the increase in the host survivability (% ILS of about ~122%), while combination treatment with AA plus CDDP showed further increase in the host survivability (ILS ~142%, Table **1**) which is about 20% more than CDDP alone treatment. The AA plus CDDP also caused maximum decrease in body weight of tumor-bearing mice which may be correlated with reduced tumor growth/ tumor volume (Figure **1**). Ascorbic acid at a nontoxic concentration, in combination with certain pharmacological agents produces a synergistic or additive effect on the growth inhibition. Taper *et al.* [38] reported that ascorbic acid increases the effectiveness of cyclophosphamide without producing new side effects.

The pro-oxidant activity of ascorbic acid is due to its ability to redox-cycle with transition metal ions, and thereby stimulates the formation of species such as superoxide, hydrogen peroxide and hydroxyl radicals. Many *in vitro* studies have shown that ascorbic acid treatment enhances the cytotoxicity of arsenic trioxide [13], doxorubicin, cisplatin, paclitaxel [27], 5-fluorouracil [39] and vincristine [40] in different cancer cell lines. The improvement of antitumor activity of cyclophosphamide *in vivo* against murine ascites Dalton's lymphoma has also been reported [10, 29]. Sarna and Bhola [41] used ascorbic acid by i.p. injection *in vivo* in combination with cisplatin and also treated tumor cells *in vitro* with cisplatin or AA plus cisplatin and then transplanted these tumor cells to animals and found an improved therapeutic efficacy in combination treatment. On the other hand, Heaney *et al.* [42] observed that pretreatment of human leukemia K562 cell lines with dehydroascorbic acid caused a dose dependent attenuation of cytotoxicity of

Table 5: Serum Alanine Aminotransferase (ALT), Aspartate Aminotransferase (AST) and Alkaline Phosphatase (ALP) Activity in Normal and Tumor-Bearing Mice Under Different Treatment Conditions

Treatment groups	ALP U/l	% control	ALT (SGPT) U/l	% control	AST (SGOT) U/l	% control
Normal	36±2.7	-	25± 2.3		60±4.3	-
Control	40.53±2.3	-	61.76±3.2	-	70.94±4.2	-
AA	35.2±2.1*	-13.15	43.5±1.9*	-29.57	62.4±3.2*	-12.03
CDDP	74.62±3.2*	84.11	84.80±2.2*	37.31	497.97±6.4*	601.96
AA+ CDDP	45.15±1.8*, #	11.39	67.65±3.3*, #	9.54	305.23±5.3*, #	330.27

Mice were sacrificed on the 4th day of CDDP treatment. Values are mean ± S.D., n =3, Student's t-test. *$P \leq 0.05$ as compared to respective control; #$P \leq 0.05$ as compared to CDDP. Normal = mice without tumor or any treatment condition; Control = Untreated tumor-bearing hosts receiving drug vehicle normal saline only; AA = Ascorbic acid; CDDP=*cis*-diamminedichloroplatinum-(II); DL = Dalton's lymphoma.

mechanistically dissimilar antineoplastic drugs such as doxorubicin, cisplatin, vincristine, methotrexate, and imatinib. Their findings indicated that it antagonizes therapeutic efficacy of these drugs by preserving mitochondrial membrane potential. However, it is noteworthy that dehydroascorbic acid is not the usual form of vitamin C which is used by people. Verrax and Buc-Calderon [15] have cited many reports in support of increase in efficacy of several chemotherapeutic drugs either *in vitro* or *in vivo*. Nonetheless, it has also been highlighted that the decrease in the activity of some agents may be the consequence of direct inactivation of the drug *in vitro* by vitamin C, as nicely described in the case of bortezomib [43].

In general the findings from various reports suggest that vitamin C together with chemotherapy drugs may overcome chemotherapy-dependent resistant cancer cells by increasing the delivery of chemotherapy drugs into cancer cells, making the tumor cell membrane more permeable to have enhanced drug delivery, stabilize p53 genes and decrease Bcl-2 and telomerase activity. It has been proposed that the enhancement of CDDP-induced tumor growth inhibition may be due to modulation of permeability of tumor cell membrane by ascorbic acid causing an increase in the uptake of cisplatin into tumor cells and making the DNA repair machinery less efficient because of more adduct formation in DNA [40].

Uncontrolled proliferation and a defect in apoptosis constitute crucial elements in the development and progression of malignancy. Apoptosis is characterized by membrane blebbing, shrinking of cells and their organelles, DNA fragmentation, and finally cell disintegration [44]. Many chemotherapeutic drugs including cisplatin have been reported to induce apoptosis in cancer cells [45]. The analysis of apoptosis in DL cells based on AO/EtBr fluorescence staining for the authentication of apoptotic features, showed that as compared to respective treatment with CDDP alone, the number of apoptotic DL cells increased after combination treatment with AA plus CDDP (Figures **2** & **3**). The treatment with AA alone also showed time dependent increase in appearance of apoptotic features in DL cells. The varied effect of ascorbic acid on the chemotherapeutic drug-induced apoptosis in different cancer cell lines has been reported. The anticancer activity of arsenic trioxide against HL-60 cells was enhanced when co-treated with AA and arsenic trioxide and it involved induction of apoptosis in these cells [13]. Ascorbate increased etoposide-induced apoptosis in HL60 cells but had no effect on etoposide-induced apoptosis in Jurkat cells. In both cell types melphalan-induced apoptosis was unaffected by intracellular ascorbate [46]. The present findings of increase in apoptotic tumor cells after combined treatment with AA plus CDDP is in conformity with earlier reports, although the exact mechanism behind it may not be clearly apparent presently. It has been reported that ascorbic acid increases the apoptosis *via* up-regulation of p53 during cisplatin treatment of human colon cancer cells [47]. In ascorbate-supplemented cells, increased cisplatin-induced apoptosis was seen, involving activation of the MLH1/c-Abl/p73 signalling cascade. The cellular response to DNA damage requires activation of MLH1, which may cooperate with the tumor-suppressor p53 gene to promote cell cycle arrest and cell death [48]. It has been reported that vitamin C (10 mM) induces apoptosis in B16 murine melanoma cells by decreasing mitochondrial membrane potential and release of cytochrome c [49].

Scanning electron microscopic (SEM) observations revealed a series of surface changes in DL cells following treatment with CDDP alone and AA plus CDDP (Figures **4** & **5**). Evenly distributed membrane projections and ruffles over the cell surface were observed in control DL cells. After 24-96 h of CDDP treatment or AA plus CDDP treatment, microvilli, membrane blebs, cell membrane fusion and deformities were noted. The formation of membrane blebs/vesicles in tumor cell observed after different treatment conditions also indicate the appearance of apoptotic features (Figures **4** & **5**).

Glutathione, an endogenous intracellular thiol-containing tripeptide (γ-L-glutamyl-L-cysteinyl-glycine) is a cellular antioxidant and has been the focus of interest in cancer chemotherapy. Under physiological conditions more than 98% of intracellular glutathione is maintained in reduced form [50]. It was observed that GSH levels in DL cells increased with tumor growth. During the growth of Ehrlich ascites tumor an increase in GSH level in tumor cells has also been reported [51]. The observed increase in GSH in DL cells may also suggest its involvement in facilitating proliferation and metabolism of tumor cells. It has been known that elevation of intracellular GSH in tumor cells is associated with mitogenic stimulation [52]. CDDP treatment of tumor-bearing mice resulted in a significant decrease in GSH levels in DL cells and combination treatment showed lowest GSH level in DL cells, although it was not significantly different from that of CDDP treatment at respective time point (Figure **7c**).

This CDDP-mediated decrease in GSH in DL cells may weaken their antioxidant defence and facilitate their killing but surviving cells may try to strengthen their antioxidant ability by recovering/ accumulating more GSH. The decrease in GSH levels in tumor cells has been suggested as a means of enhancing the cytotoxic/ genotoxic effects of chemotherapeutic agents and it may also constitute early possible signalling events in apoptotic cell death [53]. Thus, the treatment strategies involving GSH depletion may also be taken into consideration in order to maximize the therapeutic efficacy of anticancer agents.

The decrease in GSH levels in liver and kidney (Figure **7a** & **b**) after CDDP treatment could decrease protective ability which may facilitate CDDP-mediated toxic effect in these tissues. Glutathione is one of the essential compounds for maintaining cell integrity because of its reducing properties and involvement in the cell metabolism [50]. The findings from the present study revealed that as compared to control, CDDP and AA plus CDDP treatment caused a reduction in protein levels in liver, kidney and DL cells (Figure **8**). However, as compared to CDDP alone, AA plus CDDP treatment showed little improvement in protein level in liver and kidney, but there were no statistically significant difference at respective time points (Figure **8**). DNA-platinum covalent adducts have been reported to inhibit fundamental cellular processes, including replication, transcription, translation [54]. The inhibition of translation after CDDP treatment may be involved to decrease protein level [55].

Depletion in RBC leads to iron deficiency, anemia which is a frequent complication of cancer diseases. The decrease in RBCs counts and Hb content after CDDP treatment recorded in the present study may be correlated to cause decreased blood antioxidant capacity leading to anaemic condition. Cisplatin treatment has been reported to cause anemia [56], decrease in RBC, hematocrit (Hct), Hb concentration and erythropoietin (EPO) production [57]. However, an increase in the RBC counts and Hb values noted after combination treatment of AA plus CDDP (Table **2**) may suggest a protective role of AA against CDDP-induced hematotoxicity. Leukocytosis is a pathological condition often encountered in a clinical setting, usually caused by an increase in the number of neutrophils, affecting the WBC that frequently rises as a reaction to infection, chronic inflammation and cancer [58]. Our findings on neutrophils count also support this submission because as compared to normal mice without any malignancy, an increase in neutrophil count was observed in the mice bearing ascites Dalton's lymphoma (Table **3**).

Although there is a decrease in basophils and neutrophils after combination treatment, the overall increase in the number of different types of WBC (Table **3**) may suggest its significant effect on tumor growth. T lymphocytes play a key role in maintaining antitumor immunity providing an important opportunity for the immunotherapy of cancer. Thus, combination treatment of tumor-bearing mice with AA plus CDDP could be helpful in developing suitable condition in the hosts such as improved hematological values, which may be involved in decreasing CDDP-induced hematotoxicity, strengthening hosts' immunity thereby potentiating CDDP antitumor efficacy and host survivability. The consistent increase in WBC, particularly lymphocytes, may also play a role in the antitumor activity by enhancing antitumor immunity in the host.

The combination treatment with AA plus CDDP showed recovery in GSH level in both liver and kidney, showing its protective ability against CDDP induced liver and kidney damage which was confirmed by analysis of RFT and LFT. CDDP-dependent nephrotoxicity is recognized as a very complex multifactorial process. Reno-protective approaches are being discovered, but the protective effects are mostly partial, suggesting the need for combinatorial strategies [59]. The observed increase in the values of serum urea and creatinine levels in the mice treated with CDDP is an indication of appearance of CDDP-induced neprotoxicity. CDDP has been reported to increase serum urea and creatinine level in nephrotoxicity [59, 60]. However, the combination treatment with ascorbic acid and CDDP decreased this urea and creatinine level in kidney (Table **4**). This indicates that ascorbic acid has a protective role against CDDP-induced nephrotoxicity and its antioxidant property to scavenge CDDP-mediated free radicals generation may be involved in it. In another study, normal rats co-treated with cisplatin and vitamin C, depicted reduced cisplatin-induced renal toxicity and oxidative DNA damage [61].

Liver function tests (LFT) involving the assay of some marker enzymes such as ALT, AST and ALP in serum have been commonly used to detect hepatic dysfunction [62]. In present study, a significant increase in ALT, AST and ALP (Table **5**) was observed after CDDP treatment showing an indication of CDDP-induced hepatotoxicity, whereas, combination treatment with AA and CDDP caused a reduction in

ALT, AST and ALP activity as compared to CDDP treatment thereby suggesting hepatoprotective effect of AA.

In conclusion, it may be suggested that combination treatment of AA plus CDDP could be useful in enhancing CDDP-mediated therapeutic efficacy which involves induction of apoptosis in DL cells. The induction of apoptosis and decrease in GSH level in tumor cells may facilitate the lysis of tumor cells. Combination treatment of tumor-bearing mice with AA and CDDP decreased CDDP-induced hematotoxicity, hepatotoxicity and nephrotoxicity signifying its protective role, thus suggesting differential effects of the combined treatment on the cancer cells and normal tissues of the host.

CONFLICT OF INTEREST

All the authors declare that we have no conflicts of interest for the present paper.

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ABBREVIATIONS

DL = Dalton's lymphoma

GSH = reduced glutathione

RBC = red blood cells

WBC = white blood cells

DLC = differential leukocytes count

PBS = phosphate buffer saline

CDDP = *cis*-diamminedichloroplatinum-(II)

AA = ascorbic acid

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