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Biochemical changes associated with ascorbic acid–cisplatin combination therapeutic efficacy and protective effect on cisplatin-induced toxicity in tumor-bearing mice

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ABSTRACT

Cisplatin is one of the well-established anticancer drugs being used against a wide spectrum of cancers. However, full therapeutic efficacy of the drug is limited due to development of various toxicities in the host. This study examines the comparative therapeutic effectiveness and toxicities of cisplatin alone and in combination of dietary ascorbic acid (AA) in ascites Dalton's lymphoma-bearing mice. The findings show that the combination treatment of mice with ascorbic acid plus cisplatin has much better therapeutic efficacy against murine ascites Dalton's lymphoma (DL) in comparison to cisplatin alone and this may involve a decrease in reduced glutathione (GSH), catalase activity and increased lipid peroxidation (LPO) in Dalton's lymphoma tumor cells. At the same time, combination treatment indicates a protective role of ascorbic acid against cisplatin-induced tissue toxicities (side effects) in the hosts. Cisplatin-induced histopathological changes in liver, kidney and testes were decreased after combination treatment. The analysis of renal function test (RFT), liver function test (LFT) and sperm abnormalities also suggest an improvement in these parameters after combination treatment. Therefore, it may be concluded that the increased GSH level, catalase activity and decreased LPO in the tissues, *i.e.*, liver, kidney and testes after combination treatment may be involved in its protective ability against cisplatin-induced tissue toxicities in the host.

1. Introduction

Cancer and its treatment are among the most critical health issues. According to World Cancer Report released by the World Health Organization, cancer rates could further increase to 15 million new cases a year by 2020 [1]. Cancer chemotherapy has proven to be an effective treatment approach which is used either singly or in combination with surgery and/or radiotherapy.

Cis-diamminedichloroplatinum-(II) or cisplatin is a platinum-containing inorganic, square–planar complex which is a 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 [2–4]. The ability of cisplatin to react with DNA and formation of cisplatin-DNA adducts with inter- and intra-strand nuclear DNA cross-links is suggested to be the main mechanism underlying its cytotoxic effect [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 the proposal of the involvement of multistep and multilevel effects of cisplatin

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in the tumor cells/host [6]. However, the therapeutic efficacy of cisplatin is often hampered by the development of various dose-limiting side effects such as nephrotoxicity, hepatotoxicity, neurotoxicity, ototoxicity in the hosts [7] and acquired resistance by cancer cells [8]. In an attempt to overcome these impediments, the use of cisplatin in combination with some modulating agents such as WR2721 [9], quercetin [10] and cordycepin [11] have been tried with varying degree of success. Further, in an endeavour to decrease drug-induced toxicity in the host, the use of anticancer drugs such as cyclophosphamide [12], paclitaxel [13], arsenic trioxide [14] and doxorubicin [15] in combination with vitamin C have also been examined.

Ascorbic acid (L-3-ketothreohexuronic acid lactone) or vitamin C is a water soluble vitamin with antioxidant properties. Ascorbic acid is an active reducing agent involved in various biological effects and plays an important role in the metabolism and detoxification of many endogenous and exogenous compounds [16]. In spite of various reports showing good therapeutic potential of ascorbic acid against cancer [17–19] and supporting a role for increased vitamin C intake in decreasing the risk of cervical cancer [20], its definite use in cancer chemotherapy still remains inadequate [21]. Ascorbic acid has been reported to increase the efficacy of several chemotherapeutic drugs [12,22–24], though few have shown virtually no benefit from its treatment [25,26]. Some contradictory role of ascorbic acid has also been suggested in either inhibiting carcinogenesis [27–29] or enhancing carcinogenesis [30–32]. Some genotoxic effects of vitamin C in *in vitro* test systems have been demonstrated [33,34] but in *in vivo* experiments there are no genotoxic effects by vitamin C.

Thus, considering the inconsistent findings on the significance of vitamin C in cancer chemotherapy and its possible protective implication in the hosts, the present study was undertaken to evaluate the effectiveness of ascorbic acid in minimizing cisplatin-induced toxicities/side effects in Dalton's lymphoma-bearing mice. The findings exhibit that the use of ascorbic acid with cisplatin could be very useful in decreasing cisplatin-induced toxicities/side effects in the host while showing better therapeutic efficacy against murine ascites Dalton's lymphoma.

2. Materials and methods

2.1. Chemicals

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. Reduced glutathione, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Ethylenediamine tetra 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

Inbreed Swiss albino mice colony is being maintained in the laboratory under conventional conditions at room temperature of $24 \pm 2\,^{\circ}C$ with free access to food pellets (Amrut Laboratory, New Delhi) and drinking water *ad libitum*. Ascites Dalton's lymphoma is being maintained in *in vivo* in 10–12 weeks old mice by serial intraperitoneal (i.p.) transplantations of approximately 1×10^7 viable tumor cells per animal (in 0.25 ml phosphate-buffered saline, pH 7.4). Tumor transplanted hosts usually survive for 19–21 days.

The maintenance and use of the mice and the experimental protocol of the present study was approved by the Institutional Ethical Committee of North-Eastern Hill University, Shillong, India.

2.3. Drug treatment schedule and antitumor activity

Therapeutic dose of cisplatin against malignant tumors has been established to be 8–10 mg/kg body weight [2,35]. Thus, based on earlier studies [36] the dose of cisplatin (10 mg/kg body weight) was used in present studies. Similarly, the dose of AA was selected to be 1% in drinking water which has already been standardized as an effective dose [12,37]. 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 only. Group-II mice were given 1% AA through drinking water for 5 consecutive days starting from the 5th day post-tumor transplantation. Based on the volume of water intake, the AA intake was noted to be about 17.65–19.20 mg/day/per animal. Group-III mice were administered with a single dose of cisplatin (10 mg/kg body weight) on the 10th day post-tumor transplantation. Group-IV mice received AA through drinking water from the 5th day post-tumor transplantation and were administered with cisplatin (i.p., 10 mg/kg body weight) on the 10th day of tumor growth.

The anticancer efficacy was determined as percentage of average increase in life span (ILS) using the formula: $\%ILS = (T/C \times 100) - 100$, where, T and C are the mean survival days of treated and control groups of mice, respectively. The same treatment schedule was followed for the biochemical investigations in DL cells, liver, kidney and testes. Ascites fluid was collected from the peritoneal cavity of mice in different groups and was used to determine the average tumor pH using a pH meter. The ascites fluids were then centrifuged and the pellets were used as DL cells.

2.4. Total reduced glutathione estimation

Total reduced glutathione (GSH) content was determined using the method of Sedlak and Lindsay [38]. Briefly, 5% tissue homogenates of DL cells and tissues were prepared in 0.02 mol/L EDTA (pH 4.7). 100 μl of the tissue homogenate or the pure reduced form of GSH was added 1.0 ml of 0.2 mol/L Tris–EDTA buffer (pH 8.2). To this, 0.9 ml of 0.02 mol/L EDTA (pH 4.7) with 20 μl of Ellman's reagent (10 mmol/L DTNB in methanol) was added. After 30 min of incubation at room temperature, the reaction mixtures were centrifuged and the absorbance of the clear

supernatant was read at 412 nm. The results were read from a standard curve prepared from 1 mmol/L solution of reduced glutathione.

2.5. Catalase (EC 1.11.1.6) assay

Catalase activity was determined following the method of Aebi [39]. A 10% tissue homogenate was prepared in 1% triton X-100. The assay volume (3.0 ml) contained 20 μl of tissue supernatant as the enzyme source and 1.98 ml of 50 mM phosphate buffer (pH 7.0) with 1.0 ml of 30 mM H_2O_2. Catalase activity was measured by monitoring a decrease in absorbance of H_2O_2 at 240 nm. The enzyme activity was calculated using the extinction coefficient ($E_{240} = 0.0436\ mM^{-1}\ cm^{-1}$), expressed as μmol of H_2O_2 decomposed/min/mg protein.

2.6. Determination of platinum content

The ascites tumor from different treatment groups of mice were collected from the peritoneal cavity using a 1.0 ml disposable syringe and centrifuged at 5000 × *g* for 10 min, washed once in cold PBS (pH 7.4). The cell pellets (0.5 g) were digested in 5.0 ml nitric acid and few drops of hydrogen peroxide (H_2O_2) in a small clean conical flask with gentle heating to near dryness. 5.0 ml of perchloric acid was added to the digests and again heated to near dryness to remove excess nitric acid. This last step was repeated until a clear solution resulted. The digests were finally dissolved in distilled water maintaining the acidity of approximately 5% and the filtrates were stored in polypropylene bottles for platinum analysis using a labtam model 8440 m plasma lab ICP-OES emission spectrometer operated at PMT voltage 700 and wavelength 214.438 after calibrating the instrument with appropriate standard solutions.

2.7. Lipid peroxidation assay

Lipid peroxidation (LPO) level was determined as malondialdehyde (MDA) concentration following the method of Buege and Aust [40]. Briefly, 5% tissue homogenate was prepared in 0.15 M KCl. To 1 ml of the homogenate, 2 ml of TCA–TBA–HCl reagent (15% trichloroacetic acid and 0.375% thiobarbituric acid dissolved in 0.25 N HCl) was added and mixed thoroughly. After 15 min of heating in a boiling water bath, the mixture was cooled at room temperature and centrifuged (1000 × *g*, 4 °C, 10 min) to remove the precipitate. The absorbance of the clear supernatant was read at 535 nm. The MDA concentration of the tissue sample was calculated using an extinction coefficient of $1.56 \times 105\ M^{-1}\ cm^{-1}$ and expressed as nmol of MDA/mg protein.

2.8. Histopathological evaluation

Liver, kidney and testes were collected from the mice in different experimental groups on the 5th day post-cisplatin treatment and processed for histological appraisal following the methods described by Raghuramulu et al. [41]. Small tissue pieces were fixed in Bouin's fixatives (glacial acetic acid, 2.4 ml; 40% formaldehyde, 11.9 ml; saturated picric acid, 35.7 ml) for 24 h and were further routed to prepare a solid paraffin block containing the tissue. Paraffin-tissue sections (5 μm in thickness) mounted on clean glass slides were deparaffinised and stained with hematoxylin–eosin stain. The stained sections were examined and photographed under a light microscope (Leica) to analyze changes in overall cellular organization in different tissues of the hosts under different treatment conditions.

2.9. Liver function test (LFT) and renal function test (RFT)

For the assessment of LFT, changes in the activity of serum alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and for kidney (renal) functions (RFT) measurement of serum urea and creatinine levels of the hosts under different treatment conditions were determined. Blood was collected from the same mice group used for histological studies. The measurement of these biochemical parameters was done in Clinical chemistry analyzer (SYNERGY BIO-1904C) at North-East Diagnostic Centre, Shillong.

2.10. Sperm abnormality assay

After 10 days of cisplatin treatment, male mice in different groups were sacrificed and the cauda epididymis was removed and minced into pieces in physiological saline. It was then kept undisturbed for 20 min for diffusion of spermatozoa. The spermatozoa were spread on a clean slide, air-dried, fixed in absolute methanol for 15 min and then stained with 1% aqueous eosin-Y on the following day. Five hundred sperms from each mouse were examined for the abnormalities in sperm head and tail shapes following the criteria as close as possible to those established by Wyrobek and Bruce [42].

2.11. Statistical analysis

The results were expressed as the mean ± SD. Statistical significance was determined by one-way analysis of variance (ANOVA). The difference among multiple groups was analyzed by a *post hoc* (Tukey test). A *P*-value ≤0.05 was considered as statistically significant.

3. Results

3.1. Antitumor activity

Following tumor transplantation, the increase in abdominal size with sluggish movement of the animal was noted from 3rd to 4th day onwards depicting an early sign of tumor development. Control tumor transplanted mice survived for about 19–21 days. The mean survival time of mice treated with AA (group II) or cisplatin alone was significantly increased to about 34 days (ILS ~ 79%) and 42 days (ILS ~ 122%) respectively. However, the hosts survivability was further increased to more than 46 days (ILS ~ 142%) in combination treated group (Table 1, Fig. 1A and B). Significant decrease in the body weight and in tumor

Table 1
Hosts survivability, tumor size and body weight of tumor-bearing mice under different treatment conditions.

Treatment groups	Mean survival time (days)	%ILS	Tumor size (ml) on 20th day	Body weight (g) on 20th day
Group I (control)	19.2 ± 1.9	–	11.2 ± 1.2	40.3 ± 1.5
Group II (AA)	34.5 ± 3.0*	79.68	6.9 ± 1.0*	35.1 ± 1.2*
Group III (cisplatin)	42.7 ± 3.4*	122.39	4.0 ± 0.8*	32.6 ± 1.1*
Group IV (AA + cisplatin)	46.6 ± 3.7*,a,#	142.71	2.9 ± 0.7*,a,#	28.7 ± 1.1*,a,#

Results are expressed as mean ± SD. Significance of changes between control and different treated groups was tested by one way ANOVA followed by Tukey test, $n = 5$, $^{*}P \leq 0.05$ as compared to the corresponding control; $^{a}P \leq 0.05$ as compared to AA; $^{\#}P \leq 0.05$ as compared to cisplatin. Number of mice in each group = 10. Control = untreated tumor-bearing mice; AA = ascorbic acid; ILS = increase in life span.

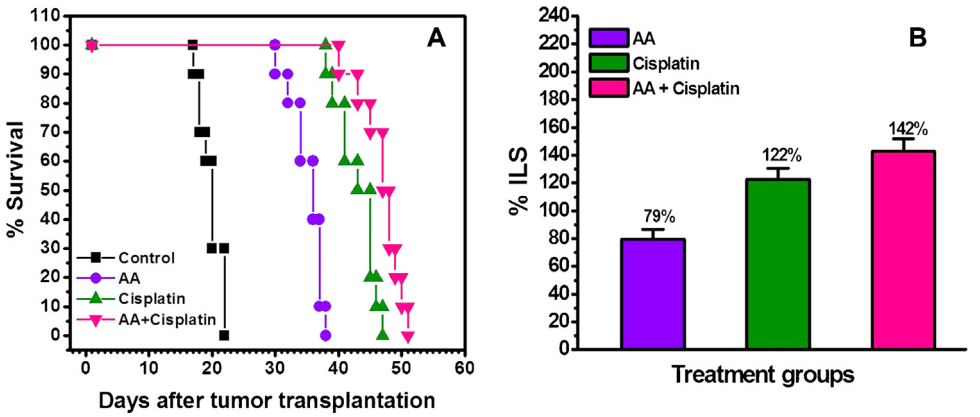

Fig. 1. Survival pattern (A), and percent increase in life span (%ILS) (B), of tumor-bearing mice in control and different treated groups. Results are expressed as mean of 5 independent experimental sets. Number of mice in each groups = 10. Control = tumor-bearing mice without AA or cisplatin treatment; AA = ascorbic acid.

size of mice was observed after combination treatment as compared to AA or cisplatin alone treatment (Table 1).

As compared to the pH of ascites tumor in control group, the pH under different treatment conditions (*i.e.*, AA, cisplatin and AA plus cisplatin) showed a time dependant decrease during 24–96 h of treatment. As compared to AA or cisplatin alone treatment group's pH, significant decrease in the tumor pH was recorded in the combination treated mice during 48–96 h (Fig. 2).

3.2. Platinum content

Intracellular accumulation of platinum (drug) in DL cells showed that the platinum content was more initially at 24 h of treatment and decreased gradually during 48–96 h in both cisplatin alone and combination treated group. As compared to AA alone or cisplatin alone treatment, platinum content was more in tumor cells after combination treatment of AA plus cisplatin (Fig. 3).

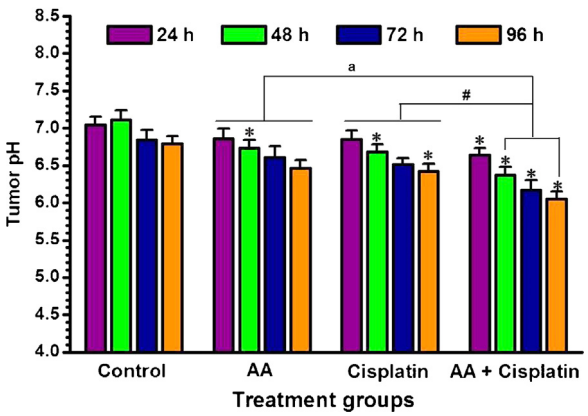

Fig. 2. Changes in the tumor pH at different treatment conditions. Results are expressed as mean ± SD. ANOVA, $n = 3$, $^{*}P \leq 0.05$ as compared to the corresponding control; $^{a}P \leq 0.05$ as compared to AA; $^{\#}P \leq 0.05$ as compared to cisplatin. Control = untreated tumor-bearing hosts; AA = ascorbic acid.

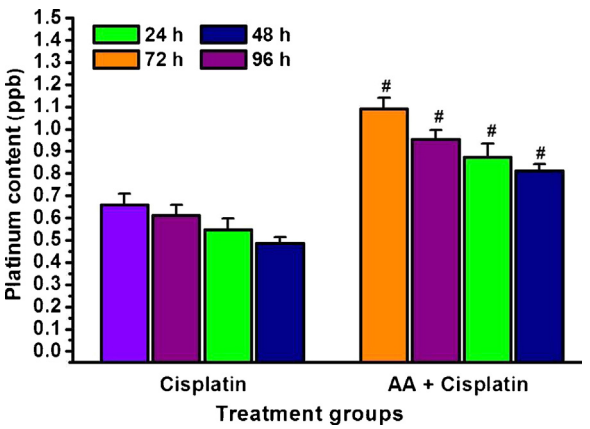

Fig. 3. Accumulation of platinum in Dalton's lymphoma (DL) cells after cisplatin or AA plus cisplatin treatment in *in vivo*. Results are expressed as mean ± SD. ANOVA, $n = 3$, $^{\#}P \leq 0.05$ as compared to cisplatin. AA = ascorbic acid; ppb = parts per billion.

Table 2
Changes in the total GSH content (μmol/g wet wt.) in the tissues of normal and tumor-bearing mice under different treatment conditions.

Treatment groups	Liver	Kidney	Testes	DL cells
Normal	13.40 ± 0.43	8.51 ± 0.73	10.61 ± 0.24	–
Control				
24 h	12.11 ± 0.46	8.43 ± 0.31	09.82 ± 0.24	4.42 ± 0.36
48 h	11.58 ± 0.33	8.19 ± 0.23	09.58 ± 0.27	4.35 ± 0.22
72 h	11.33 ± 0.45	7.93 ± 0.36	09.23 ± 0.28	4.31 ± 0.19
96 h	11.19 ± 0.19	7.85 ± 0.22	09.15 ± 0.30	4.28 ± 0.21
AA treated				
24 h	11.83 ± 0.60*	6.47 ± 0.35*	08.28 ± 0.23*	3.31 ± 0.27*
48 h	11.38 ± 0.22	6.65 ± 0.28*	08.36 ± 0.31*	3.60 ± 0.25*
72 h	10.19 ± 0.32*	7.38 ± 0.34	08.78 ± 0.35	4.02 ± 0.19
96 h	11.03 ± 0.31	7.59 ± 0.32	08.85 ± 0.29	4.18 ± 0.26
Cisplatin treated				
24 h	10.87 ± 0.29*	7.04 ± 0.35*	08.46 ± 0.27*	3.40 ± 0.23*
48 h	10.52 ± 0.26*	6.74 ± 0.34*	08.53 ± 0.22*	3.65 ± 0.17*
72 h	09.91 ± 0.34*	7.16 ± 0.28*	08.74 ± 0.25*	3.85 ± 0.18*
96 h	10.31 ± 0.25*	7.39 ± 0.24	09.06 ± 0.29	4.13 ± 0.18
AA + cisplatin treated				
24 h	10.55 ± 0.25*,a	6.76 ± 0.31*,a	09.17 ± 0.24*,a,#	3.11 ± 0.17*
48 h	10.69 ± 0.23*,a	6.90 ± 0.30*	09.04 ± 0.34*,#	3.39 ± 0.21*
72 h	10.78 ± 0.25*,a,#	7.62 ± 0.25#	09.31 ± 0.27*,#	3.54 ± 0.16*,#
96 h	10.93 ± 0.27#	7.78 ± 0.24#	09.50 ± 0.30	3.83 ± 0.19*,a,#

Values represent mean ± SD, $n = 3$. Significance of difference was tested by ANOVA, followed by Tukey test. *$P \leq 0.05$ compared to the corresponding control; a$P \leq 0.05$ as compared to AA; #$P \leq 0.05$ as compared to cisplatin. Normal = hosts without tumor or any treatment condition; control = untreated tumor-bearing mice; AA = ascorbic acid; DL = Dalton's lymphoma.

3.3. Reduced glutathione (GSH)

As compared to the GSH level in the tissues of normal mice, the GSH level decreased in the corresponding tissues of tumor-bearing control mice. AA alone treatment showed a decrease in GSH level in different tissues and DL cells during 24–48 h of treatment. Cisplatin treatment of tumor-bearing mice resulted in a significant decrease in GSH level in liver during 24–96 h and in kidney, testes and DL cells during 24–72 h of treatment. As compared to AA alone, combination treatment caused significant increase in GSH level in liver (72 h), kidney (24 h) and testes (24 h) while a decrease was noted in DL cells at 96 h of treatment. As compared to cisplatin alone, AA plus cisplatin treatment caused a significant increase in GSH levels in liver and kidney at 72–96 h, and in testes during 24–72 h of treatment. However, DL cells showed a significant decrease in GSH levels at 24–72 h of treatment (Table 2).

3.4. Catalase (EC 1.11.1.6) activity

As compared to corresponding control, AA alone treatment showed a significant decrease in CAT activity in liver and kidney at 72 h and in DL cells at 24–72 h of treatment respectively. Cisplatin treatment of tumor-bearing mice caused a time dependent decrease in catalase activity in liver, kidney and DL cells at 24–96 h and in testes during 24–72 h of treatment (Fig. 4A–D). As compared to cisplatin alone, combination treatment of mice caused an increase in catalase activity in liver and kidney during 24–72 h and in testes at 72 h (Fig. 4A–D). However, significant decrease in catalase activity in DL cells was noted at 24–72 h of combination treatment (Fig. 4A–D).

3.5. Lipid peroxidation (LPO)

As compared to malondialdehyde (MDA) level, *i.e.*, lipid peroxidation in the liver, kidney and testes of normal mice, LPO level increased in liver and kidney of tumor-bearing mice while a decrease was observed in testes. As compared to control cisplatin treatment caused an increase in LPO in liver, kidney and DL cell at different time points. However, in testes decrease in LPO was observed after cisplatin treatment (Table 3). As compared to AA, combination treatment caused a decrease in LPO in liver and kidney while an increase was noted in DL cells at 24–48 h. As compared to cisplatin alone, combination treatment of mice caused a decrease in the LPO level in liver during 24–96 h and in kidney, testes and DL cells during 48–96 h of treatment (Table 3).

3.6. Histopathological changes

Histopathological examination of kidney in control mice showed normal renal glomeruli and tubules having intact epithelial cells. Ascorbic acid treatment showed almost similar histological features as observed for control group (Fig. 5A and B). However, cisplatin treatment of mice caused close to grade 2 damages in kidney represented by glomerular atrophy, infiltration of cells and tubular congestions (Fig. 5C) which supports the view that cisplatin alone treatment caused nephrotoxicity in the host. However, combination treatment of AA plus cisplatin in the hosts led to a reduction in damages close to grade 1 in renal tubular cells with lesser glomerular damages (Fig. 5D, Table 4).

Liver sections from control mice showed normal arrangement of hepatocytes and proper central vein

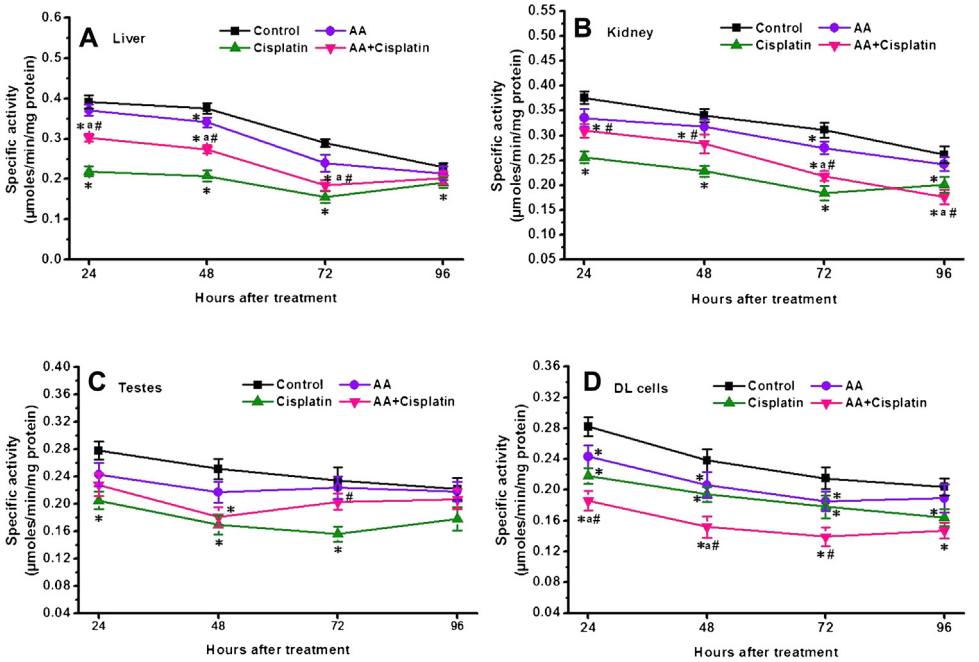

Fig. 4. Changes in the activity of catalase (μmol/min/mg protein) in the liver (A), kidney (B), testes (C) and DL cells (D), at different treatment conditions. Results are expressed as mean ± SD. ANOVA, $n = 3$, $^{*}P \leq 0.05$ as compared to the corresponding control; $^{a}P \leq 0.05$ as compared to AA; $^{\#}P \leq 0.05$ as compared to cisplatin. Control = untreated tumor-bearing mice; AA = ascorbic acid.

Table 3
Changes in the level of malondialdehyde concentration (nmol/mg protein) reflecting lipid peroxidation in the tissues of normal and tumor-bearing mice under different treatment conditions.

Treatment groups	Liver	Kidney	Testes	DL cells
Normal	0.309 ± 0.018	0.291 ± 0.060	0.447 ± 0.029	–
Control				
24 h	0.347 ± 0.043	0.361 ± 0.025	0.376 ± 0.021	0.124 ± 0.019
48 h	0.356 ± 0.025	0.387 ± 0.030	0.383 ± 0.022	0.120 ± 0.016
72 h	0.407 ± 0.031	0.411 ± 0.021	0.394 ± 0.025	0.117 ± 0.015
96 h	0.425 ± 0.032	0.441 ± 0.028	0.370 ± 0.032	0.106 ± 0.016
AA treated				
24 h	0.329 ± 0.044	0.351 ± 0.025	0.184 ± 0.025*	0.142 ± 0.019
48 h	0.332 ± 0.031	0.419 ± 0.028	0.169 ± 0.030*	0.156 ± 0.024
72 h	0.383 ± 0.025	0.403 ± 0.031	0.155 ± 0.032*	0.161 ± 0.019*
96 h	0.391 ± 0.035	0.372 ± 0.031*	0.162 ± 0.026*	0.158 ± 0.020*
Cisplatin treated				
24 h	0.355 ± 0.028	0.452 ± 0.025*	0.248 ± 0.031*	0.196 ± 0.020*
48 h	0.396 ± 0.030*	0.469 ± 0.030*	0.269 ± 0.030*	0.209 ± 0.015*
72 h	0.474 ± 0.031*	0.428 ± 0.032	0.323 ± 0.041*	0.202 ± 0.018*
96 h	0.443 ± 0.033	0.397 ± 0.023	0.315 ± 0.024*	0.203 ± 0.017*
AA + cisplatin treated				
24 h	0.271 ± 0.026$^{*,a,\#}$	0.414 ± 0.023$^{*,\#}$	0.219 ± 0.031*	0.167 ± 0.017^{*,a}
48 h	0.314 ± 0.024$^{\#}$	0.391 ± 0.031$^{\#}$	0.197 ± 0.021$^{*,\#}$	0.179 ± 0.018$^{*,a,\#}$
72 h	0.352 ± 0.032$^{*,\#}$	0.360 ± 0.028$^{*,\#}$	0.189 ± 0.029$^{*,\#}$	0.154 ± 0.020$^{\#}$
96 h	0.379 ± 0.030$^{\#}$	0.344 ± 0.030*	0.206 ± 0.023*	0.148 ± 0.018$^{*,\#}$

Values represent mean ± SD, $n = 3$. Significance of difference was tested by ANOVA followed by Tukey test, $^{*}P \leq 0.05$, significance as compared to the corresponding control; $^{a}P \leq 0.05$ as compared to AA; $^{\#}P \leq 0.05$ as compared to cisplatin. Normal = hosts without tumor or any treatment condition; control = untreated tumor-bearing mice; AA = ascorbic acid; DL = Dalton's lymphoma.

(Fig. 6A). As compared to control, AA treatment did not show much change in the histological features of liver (Fig. 6B). In contrast, treatment of mice with cisplatin exhibited severe hepatotoxicity characterized by diffused sinusoidal distortion, congestion in central vein and marked zonal hepatocytes damages (Fig. 6C). However, combination treatment of AA plus cisplatin in the hosts showed mild to moderate damage of hepatocytes indicating recovery in the damaged cells (Fig. 6D, Table 4).

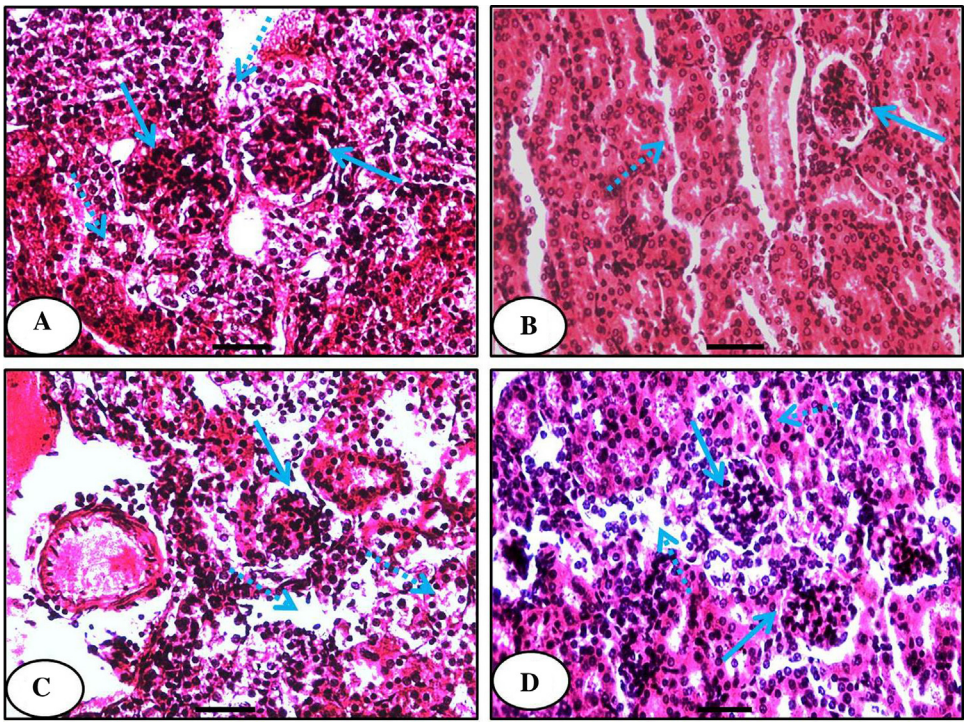

Fig. 5. Histological features of kidney from mice in control and different treated groups. (A) Control and (B) AA alone showing intact glomerular (regular arrow) and tubular (dotted arrow) arrangement, (C) cisplatin alone treatment, showing damaged tubules (dotted arrow), congested vein and degenerate glomeruli, and (D) ascorbic acid (AA) plus cisplatin treatment, showing reduced damages in glomerular (regular arrow) and tubular cells (dotted arrow). Scale bar = 50 μm.

Table 4
Histological damages graded according to pathological characterization for kidney, liver and testes of tumor-bearing mice under different treatment conditions.

Tissue	Parameters	Treatments			
		Control	AA	Cisplatin	AA + cisplatin
Kidney	Tubular congestion	–	–	++++	++
	Tubular cast	–	–	++	+
	Epithelial disquamation	–	–	+++	+
	Glomerular congestion	–	–	+++	+
	Blood vessel congestion	–	–	++++	++
	Hyperaemia of medullary part	–	–	++	+
Liver	Haemorrhage	–	–	++++	+
	Perinuclear clumping of cytoplasm	–	–	+++	+
	Hepatic vacuolation	–	–	+++	++
	PMN infiltration	–	–	+++	+
Testes	Tubular atrophy	–	–	+++	+
	Sertoli cells vacuolation and spermiogenic abnormalities	–	–	++++	++
	Leydig cells hypertrophy	–	–	+++	++
	Degeneration/regression in leydig cells	–	–	++++	++

–, Nil; +, low/minimal (<12%); ++, mild/moderate (<20%); +++, high (<40%); ++++, very high/severe (>40%); PMN, polymorphonuclear leukocytes.

Histopathological observations of testes of control mice revealed well-defined seminiferous tubules, spermatogenic cells and leydig cells in interstitial tissue (Fig. 7A). AA alone treatment also showed similar regular pattern of cellular morphology with normal spermatozoa seen lying in the lumen of seminiferous tubules (Fig. 7B). Cisplatin alone treatment caused vacuolization in sertoli cells or dense granules in the cytoplasm, damaged seminiferous tubules and degeneration and regression of leydig cells (Fig. 7C), while combination treatment of AA plus cisplatin in the hosts showed an improvement in spermatogenic cells, reduced vacuolated tubules and less deranged spermatogonial mass (Fig. 7D, Table 4).

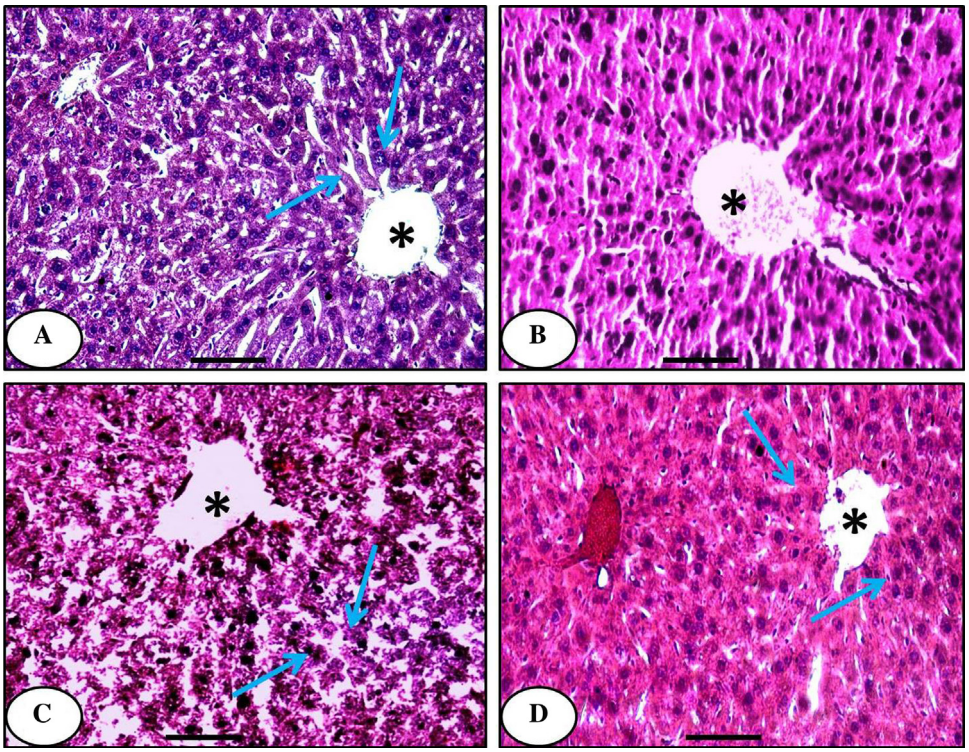

Fig. 6. Histological features of liver from mice in control and different treated groups. (A) Control, showing normal hepatocytes arrangement (regular arrow) and proper central vein (asterix); (B) AA alone treatment, showing features as that of control; (C) cisplatin alone treatment, showing locational hepatocytes damage (regular arrow) and congestion in central vein (asterix); (D) ascorbic acid (AA) plus cisplatin treatment, showing recovery of damaged hepatic cells (regular arrow). Scale bar = 50 μm.

3.7. Renal function test (RFT) and liver function test (LFT)

3.7.1. RFT

Serum urea and creatinine level were studied to further monitor the renal toxicity in the hosts. AA alone treatment also did not show any appreciable variation as compared to the control. Cisplatin-treated mice showed significant elevation in serum urea and creatinine levels. The increase in serum urea and creatinine level was about ~270% and ~302%, respectively (Fig. 8). However, as compared to cisplatin alone, treatment of tumor-bearing mice with AA plus cisplatin significantly brought down the elevated levels of serum urea (~67%) and creatinine (~66%) (Fig. 8).

3.7.2. LFT

The activity of serum alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were studied to assess hepatotoxicity in the hosts under different treatment conditions. Here also, AA alone treatment did not show any appreciable variation. Significant increase in ALP (~84%), ALT (~37%) and AST (~601%) activities were observed after cisplatin alone treatment (Fig. 8). However, as compared to cisplatin alone, combination treatment caused significant decrease in ALP (~11%), ALT (~9%) and AST (~330%) activity (Fig. 8).

3.8. Sperm abnormality assay

Comparative analysis of sperm abnormalities in mice treated with cisplatin alone showed maximum increase (~34%) in the frequency of sperm abnormalities. AA treatment did not show any significant changes in the incidence of sperm abnormalities. However, pre-treatment of mice with AA in combination treatment significantly decreased (~20%) the frequency of cisplatin-induced sperm abnormalities in the hosts (Table 5). Various types/shapes of observed sperm abnormalities included hookless head, looping mid-piece, microhead, balloon-like head, double tailed, incorrect head–neck connection, diffused head, banana head, amorphous head, *etc.* (Table 5).

4. Discussion

Cisplatin is one of the well-known potent cancer chemotherapeutic agents, displaying clinical usefulness against a wide variety of cancers [8]. Various reports suggest that combination of different drugs/agents could be more effective in cancer therapy and at the same time reducing side effects in the host. Ascites Dalton's lymphoma has been used as a common experimental malignant tumor to evaluate the antitumor activity of different drugs such as plant extracts [43,44], cantharidin [45], cyclophosphamide [12], chlorambucil [46] and cisplatin [35]. The findings from the present studies showed that the Dalton's lymphoma (DL) ascites-bearing mice treated with AA or cisplatin alone depicted the increase in mean survival time of the hosts to about 79% and 122%, respectively while combination treatment of AA plus cisplatin it further increased to about 142% (Table 1, Fig. 1B). The increase in

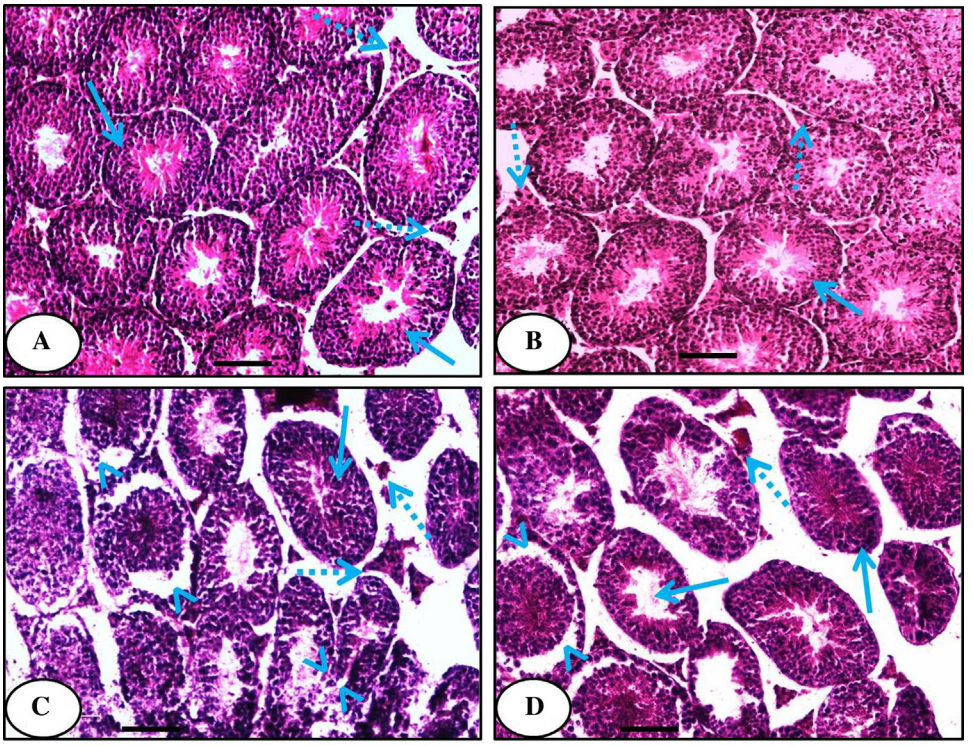

Fig. 7. Histological features of testes in control and different treated groups. (A) Control, showing well-defined seminiferous tubules (regular arrow) and leydig cell (dotted arrow); (B) AA alone treatment, showing normal spermatozoa (regular arrow); (C) cisplatin alone treatment, showing damaged tubules with vacuolated sertoli cells (arrow head) and degenerated leydig cells (dotted arrow); (D) AA plus cisplatin treatment, showing reduced vacuolated tubules (arrow head) with less deranged spermatogonial mass (regular arrow) and leydig cells damage (dotted arrow). Scale bar = 50 μm.

survivability of the hosts was accompanied by a decrease in tumor size as well as body weights in different treated groups showing maximum decrease in tumor size and body weight after combination treatment (Table 1). This may suggest that as compared to AA or cisplatin alone, AA plus cisplatin combination treatment could be a better therapeutic strategy against murine ascites Dalton's lymphoma. Combination of ascorbic acid with other pharmacological

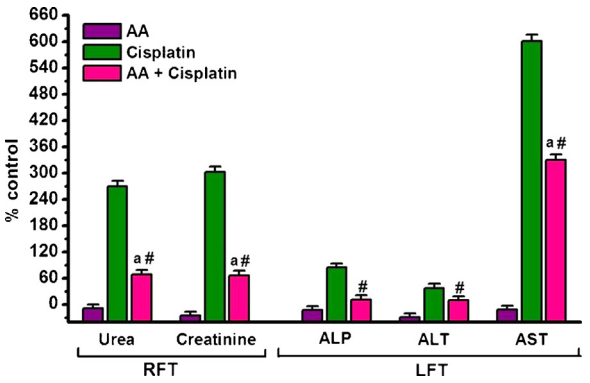

Fig. 8. Changes in the parameters of renal function test (RFT) and liver function test (LFT) in tumor-bearing mice at different treatment conditions. Results are expressed as mean ± SD, $n = 3$. ANOVA, $^{a}P \leq 0.05$ as compared to AA; $^{\#}P \leq 0.05$ as compared to cisplatin. AA = ascorbic acid, ALP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase.

agents such as cyclophosphamide [12] and chlorambucil [46] have also been reported to exhibit enhanced antitumor activity against ascites Dalton's lymphoma as compared to drug alone. Similar findings have been reported using other cancer models also where it has been observed that pharmacological doses of ascorbic acid enhanced the effects of arsenic trioxide on ovarian cancer cells [47], gemcitabine on pancreatic cancer cells [48] and combination treatment of gemcitabine and epigallocatechin-3-gallate (EGCG) on mesothelioma cells [49]. The combinatorial treatment of ascorbic acid and paclitaxel against H1299 (a non-small cell lung cancer cell line) and BALB/c mice implanted with or without sarcoma 180 cancer cells showed that the anticancer effects of the combinational treatment were up to 1.7-fold higher than those of single-agent paclitaxel treatment [13].

Extracellular acidosis is frequently associated with tumor growth. Due to elevated production of lactic acid as a result of anaerobic glycolysis, the microenvironment in cancer cells are known to be acidic and intrinsically hypoxic relative to normal tissues [50]. Such acidic environment and also the nature of drugs greatly influence the response of cancer cells to various treatments [51]. For instance, the acidic extracellular pH increases the cellular uptake of weakly acidic drugs such as cyclophosphamide and cisplatin, thereby increasing the effect of these drugs, whereas the acidic extracellular pH retards the uptake of weakly basic drugs such as doxorubicin and vinblastine, thereby reducing the effect of the drugs [52]. Here also, the decrease

Table 5
Quantitative analysis of various types of sperm abnormalities induced after different treatment conditions.

Treatment groups	Hookless	Looping mid-piece	Microhead	Balloon-like	Double tailed	Incorrect head–neck connection	Diffused head	Banana head	Amorphous head	Total abnormal sperms
AA treated	1	0	1	1	0	0	0	2	2	7
Cisplatin treated	6	1	3	2	2	1	3	9	7	34
AA + cisplatin treated	3	1	2	1	1	0	1	5	6	20

Data are shown over percent control. Sperm analysis was carried out on 10th day of cisplatin treatment. AA = ascorbic acid.

in tumor pH noted especially after combination treatment of AA plus cisplatin (Fig. 2) may facilitate the antitumor activity of cisplatin and resulting in an increase in host survivability (Fig. 1). It has been established that hypoxic environment up regulates a number of transcription factors such as HIF-1 [53]. HIF-1 has been demonstrated to activate transcription of as many as 70 genes including glucose transporters and glycolytic enzymes, which may account for the increased anaerobic glycolysis and resultant acidification of tumors under a hypoxic environment [54]. It has also been observed that exposure of tumor cells to a low pH medium elevates p53 and p21 expression [55].

The cytotoxic activity of cisplatin may also be correlated with the amounts of platinum and its binding to DNA [56]. The accumulation of platinum (drug) in DL cells was noted to be more during 24 h which decreased later during 48–96 h of different treatment conditions (Fig. 3). This may be due to its export from the cells and may also be correlated with the recovery of GSH levels during the later period (Table 2). The availability of more drugs during initial stage of treatment may be thought to give rise to various metabolic dysfunctions directly or indirectly related to cisplatin cytotoxicity which may be partially repaired or retained within the DL cells, leading to tumor regression. It was observed that combination treatment significantly increased the amount of platinum (drug) in DL cells (Fig. 3) which could be an important contributory factor in resulting better cytotoxic effects during combination treatment against murine ascites Dalton's lymphoma and it is aided by decreased GSH levels in tumor cells as observed in the present study (Table 2). Combination treatment of AA and H_2O_2 has been reported to cause a significant decrease in GSH levels in the mouse neuroblastoma cells leading to effective death of cancer cells [57]. Increased cisplatin-induced apoptosis has been reported in ascorbate-supplemented cells by upregulation of MLH1 and p73 gene [58]. It has been suggested that ascorbic acid increases tumor cell membrane permeability to chemotherapeutic drugs and helps to increase the uptake of chemotherapy drugs into cancer cells [23]. It has also been suggested that ascorbic acid-mediated sensitization of cancer cells to chemotherapy treatments involves enhancement of membrane transport systems or the activation of tumor suppressor genes [23]. Therefore, the observed further decrease in GSH level in tumor cells after combination treatment of AA plus cisplatin (Table 2) as compared to AA or cisplatin alone may help to enhance its cytotoxic effects, thereby increasing tumor cells' susceptibility to cell death. In fact changes in the intracellular GSH levels could also affect the drug uptake by DL cells. In study using freshly isolated peripheral blood mononuclear cells, it has been reported that the increased intracellular GSH concentration is correlated with decreased platinum-DNA binding [59]. It has been well established that cisplatin is detoxified by conjugation with GSH followed by the export of the conjugate by the GS-X pump [60]. Hence, the enhancement of cisplatin-induced tumor 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 due to more adduct formation in the DNA which could also be assisted by decreased GSH level (Table 2). Depletion of cellular GSH by buthionine sulfoximine (BSO) has been shown to sensitize tumor cells to oxidative stress, irradiation and certain chemotherapeutic agents' in *in vitro* [61,62]. Elevation of GSH in cellular resistance to platinum agents has been reported in several human and murine tumor cell lines [63].

Catalase is an endogenous antioxidant enzyme that neutralizes ROS by converting H_2O_2 into H_2O and O_2 and thus protects cells from oxidative damage [64]. As compared to the corresponding control or AA treatment, it was observed that cisplatin alone or AA plus cisplatin treatment notably caused a significant decrease in catalase activity in DL cells (Fig. 4D) which may result in its decreased ability to scavenge toxic hydrogen peroxides thereby increase production of ROS and oxidative stress and may also cause enhanced LPO (Table 3) in DL cells to facilitate its death. The findings are in agreement with the earlier reports on induced LPO and decreased catalase activity by treatment with anticancer drugs [65,66]. Negahdar et al. [67] reported that low levels of catalase activity in breast cancer resulted in higher production of ROS due to inadequate enzyme activity to detoxify high levels of hydrogen peroxide thereby, leading to formation of hydroxyl radicals. Low levels may be due to treatment by anticancer drugs which reduces antioxidants and induces oxidative stress [68].

Histopathological evaluations are commonly used for detecting organ-specific effects related to chemical exposure [69,70]. Ascorbic acid treatment showed almost similar histological features of kidney, liver and testis as observed for control group (Figs. 5–7, Table 4). Analysis of comparative histological changes in kidney revealed that cisplatin treatment caused destruction of the renal tubular cells close to grade 2 damages (Fig. 5C, Table 4), and in liver it showed sinusoidal distortion and marked zonal hepatocytes damage (Fig. 6C, Table 4). Some studies have suggested that oxidative stress plays an important role

in cisplatin-induced tissue damage [71–73] resulting in enhanced production of ROS by decreasing the activity of antioxidant enzymes [74] and by depleting intracellular concentrations of GSH [75]. The decrease in catalase activity (Fig. 4) and GSH concentration (Table 2) particularly in kidney and liver after cisplatin treatment as observed in the present study may be manifested in the enhanced oxidative stress and pathogenesis of cisplatin-induced renal injury and hepatocytes damage. The involvement of oxidative stress in cisplatin-induced toxicity may be further supported by the fact that many antioxidants have been reported to prevent cisplatin-induced nephrotoxicity [76] and hepatotoxicity [77]. Agents such as superoxide dismutase, dimethylthiourea and GSH have been shown to reduce the degree of renal failure and tubular cell damage when administered simultaneously with cisplatin in rats [78]. In the present study, AA alone did not show much changes in the histological features, however AA administration prior to cisplatin injection showed amelioration in the histopathological changes showing reduced destruction of renal tubular cells with lesser glomerular damage, thus inferring nephroprotectivity (Fig. 5D), and mild to moderate damage of hepatic cell indicating recovery of the altered tissue (Fig. 6D). Atasayar et al. [79] demonstrated that combined treatment of vitamin C and E with single acute dose (toxic dose) of cisplatin is able to normalize the histopathological alteration induced by cisplatin on kidney when compared with cisplatin alone treated group. The mechanisms by which ascorbic acid decreases the hepatotoxicity induced by cisplatin, is embodied in the fact that ascorbic acid might ameliorate the oxidative damage by decreasing LPO and altering antioxidant defense system [80,81].

Cisplatin-induced histopathological changes showing toxicity in kidney and liver were substantiated by specific biochemical changes in these tissues. Cisplatin has been reported to increase serum urea and creatinine level in nephrotoxicity [82,83]. Cisplatin treatment caused an increase in serum creatinine and urea levels (Fig. 8), with increased lipid peroxides (Table 3) depletion of GSH (Table 2) and decrease in catalase activities (Fig. 4A–C). ROS have been implicated as important mediators of the acute renal failure induced by cisplatin and other toxic agents, such as gentamicin and cyclosporin A [84]. The observed increase in serum creatinine and urea levels (Fig. 8) may also be due to decreased glomerular filtration rate or increased ROS. It has been reported that administration of cisplatin to rats incites nephrotoxicity, which was correlated with increased creatinine and urea in plasma [65,85]. However, combination treatment with AA plus cisplatin decreased the serum urea and creatinine level (Fig. 8), indicating that pre-treatment with ascorbic acid has a protective role against cisplatin-induced nephrotoxicity. Its antioxidant property to scavenge cisplatin-mediated free radicals generation may also be involved in it.

Liver function tests (LFT) have been commonly used to detect hepatic dysfunction [86] where assay of serum ALT, ALP and AST are the most frequently used hepatocellular markers to analyze hepatocellular injury. In the present study, hepatotoxicity induced by cisplatin was recognized by the marked elevation in serum ALP, ALT and AST enzymes activities (Fig. 8). However, these cisplatin-induced elevations in enzyme activities were significantly attenuated by AA pre-treatment in combination group thereby suggesting its hepatoprotective effect.

As an antioxidant agent, ascorbic acid and elevated level of GSH in the tissues under combination treatment condition may have a role to scavenge the reactive free radicals in these tissues. Other studies have equally shown the protective role of ascorbic acid and other vitamins in hepatic oxidative damage [87,88]. Thus, results of the present study suggests ascorbic acid's ameliorating effects to be likely mediated *via* inhibition of free radicals generation and/or free radical scavenging activity. The observed betterment in histopathology as well as RFT and LFT along with the recovery in GSH level in liver and kidney after combination treatment with AA plus cisplatin should be important in its protective ability against cisplatin-induced liver and kidney damage.

It is well known that, cytotoxic drugs depress spermatogenesis in mammals [89] by causing the death of the developing germ cells in the seminiferous tubules. Cisplatin based chemotherapy have led to persistent impairment of fertility and leydig cell function in the majority of patients with testicular cancer accompanied by significant reduction in sperm production [90]. In the present study, degenerations in seminiferous tubules, intercellular disassociation of germ cells, vacuolization in sertoli cells and reduction in leydig cells were seen following cisplatin treatment (Fig. 7C). The presence of vacuoles in seminiferous tubules is an indicator of sertoli cells damage owing to microtubule disruption [91,92]. Combination treatment of mice with AA plus cisplatin revealed reduced vacuolated tubules and less deranged spermatogonial mass (Fig. 7D, Table 4). Other researchers have similarly reported that combined pre-treatment with antioxidants including vitamin C can alleviate the damage caused to testis by lindane [93]. Ascorbic acid has long been known to participate in spermatogenesis process of rodents and ameliorate oxidative stress related to testicular impairments in animals [94]. Moreover, dietary AA was found to protect against endogenous oxidative DNA damage in human sperms [95]. Thus, ascorbic acid pre-treatment may be thought to enhance the defense system during stress to combat tissue destruction and safeguard cellular structure and function thereby, ameliorating cisplatin-induced testicular toxicity, as observed for kidney and liver, in the hosts. Ascorbic acid has also been reported to be effective in the protection of aluminium-induced tissue toxicity in rabbits [96].

Cisplatin has been considered as a potent mutagen causing formation of abnormal male germ cells [97,98]. In the present study, it was observed that treatment with cisplatin caused a significant increase in sperm abnormalities in tumor-bearing male mice for 10 days when treated alone and in combination with AA (Table 5). Among different treatment conditions, cisplatin caused maximum (~34%) abnormalities in the sperms suggesting that cisplatin could reach the germ line cells and indicates its potentiality as germ cell mutagen also. The development in sperm abnormalities may indicate that cisplatin induces the DNA damage in germ cells leading to altered sperm morphology [99]. Several studies indicate that various

species of ROS generated through metal catalysis potentially interact with gene strands causing mutations, thereby inducing changes in sperm morphology [100–102]. Therefore, the formation of abnormal sperm as observed in the present study may also be an oxidative stress dependent phenomenon induced by cisplatin. However, combination treatment with AA showed a significant decrease (~20%) in the sperms abnormalities (Table 5). Ayinde et al. [103] reported that daily consumption of vitamin C could be useful in lowering oxidative stress and spermatozoa deformations in lead exposed albino rats. It has also been reported that co-administration of speman with cisplatin showed significant improvement on the sperm quantity and quality along with enhanced steroidogenesis in mice [104]. In fact the use of another antioxidant, *i.e.*, melatonin in combination with cisplatin has also shown chemoprotective effect against cisplatin-induced testicular toxicity in rats [99] which also supports the present findings.

Therefore, the observed cisplatin-mediated decrease in GSH level in the tissues, *i.e.*, liver, kidney and testes (Table 2) may have a role in altering cellular antioxidant machinery and in lessening the protective mechanism of tissues which may facilitate cisplatin-mediated toxic effect in these tissues. Thus, the decreased level of GSH observed in testes of tumor bearing mice (Table 2) should also be helping to enhance cisplatin's toxic/mutagenic effects in testes. Although combination treatment caused a decrease in the GSH level in the tissues during 24–72 h, GSH level was restored to approximately control levels at 96 h (Table 2). As GSH is known to play a role in detoxifying many reactive metabolites, the increased GSH levels in the tissues after combination treatment with AA plus cisplatin as compared to cisplatin alone, could also represent a protective mechanism in response to various toxic radicals, thereby, suggesting the possible involvement of cellular GSH as a mechanistic step in AA-mediated protection against cisplatin-induced toxicity. Nagyová and Ginter [105] have also reported the protective role of ascorbic acid in 2,4-dichlorophenol induced teratogenic/carcinogenic toxicity along with significantly increased liver ascorbic acid and GSH levels.

Lipid peroxidation (LPO) is a key process in many pathological events and it is induced by oxidative stress. It is regarded as one of the fundamental mechanisms of cellular damage caused by free radicals having reacted with lipids causing peroxidation that eventually results in the release of products such as malondialdehyde (MDA), hydrogen peroxide (H_2O_2) and hydroxyl radicals. All cellular components are susceptible to attack by ROS, particularly by hydroxyl radicals. Cisplatin treatment is associated with induction of oxidative stress by generation of free radicals and ROS [106,107]. In the present study, the increased LPO level in the DL cells and tissues after cisplatin treatment (Table 3) may be attributed to antitumor activity in DL cells and toxicity in tissues. However, in combination treatment, co-administration of AA and cisplatin to mice significantly decreased the level of LPO in liver, kidney and testes (Table 3). The observed increase in catalase activity in the tissues after combination treatment (Fig. 4A–C) may also be involved to lower LPO level and play a compensatory regulatory role in decreasing cisplatin alone-induced oxidative stress and toxicities in the tissues of the host. These results imply that the increased activities of catalase as well as increased concentration of reduced GSH in the tissues after combination treatment of ascorbic acid plus cisplatin should be very helpful to decrease cisplatin-induced toxicities involving excess hydrogen peroxide production and/or oxidative stress in the tissues of the host.

5. Conclusions

From the present findings, it may be suggested that combination chemotherapy with dietary ascorbic acid and cisplatin could be very useful in augmenting cisplatin-mediated therapeutic efficacy against murine ascites Dalton's lymphoma and other cancers in general, and at the same time in decreasing cisplatin-induced toxicities (nephrotoxicity, hepatotoxicity and testes/sperms abnormalities) in the hosts. However, the details of the molecular mechanisms behind better therapeutic efficacy and the protective function of ascorbic acid in combination with cisplatin treatment in the hosts needs further exploration.

Conflict of interest

The authors have no conflict of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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