# Chlorambucil and ascorbic acid-mediated anticancer activity and hematological toxicity in Dalton's ascites lymphoma-bearing mice

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Chlorambucil is an anticancer drug with alkylating and immunosuppressive activities. Considering various reports on the possible antioxidant/protective functions of ascorbic acid (vitamin C), it was aimed at to explore the modulatory effect of ascorbic acid on therapeutic efficacy and toxicity induced by chlorambucil. Dalton's ascites lymphoma tumor serially maintained in Swiss albino mice were used for the present experiments. The result of antitumor activity showed that combination treatment with ascorbic acid and chlorambucil exhibited enhanced antitumor activity with 170% increase in life span (ILS), which is significantly higher as compared to chlorambucil alone (ILS 140%). Analysis of apoptosis in Dalton's lymphoma tumor cells revealed a significantly higher apoptotic index after combination treatment as compared to chlorambucil alone. Blood hemoglobin content, erythrocytes and leukocytes counts were decreased after chlorambucil treatment, however, overall recovery in these hematological values was noted after combination treatment. Chlorambucil treatment also caused morphological abnormalities in red blood cells, majority of which include acanthocytes, burr and microcystis. Combination treatment of mice with ascorbic acid plus chlorambucil showed less histopathological changes in kidney as compared to chlorambucil treatment alone, thus, ascorbic acid is effective in reducing chlorambucil-induced renal toxicity in the hosts. Based on the results, for further development, hopefully into the clinical usage, the administration of ascorbic acid in combination with chlorambucil may be recommended.

Keywords: Apoptosis, Ascorbic acid, Chlorambucil, Dalton's lymphoma, Hematotoxicity

Chlorambucil [4{bis(2chlorethyl)amino}benzenebutyric acid], is an alkylating anticancer drug which was first introduced for the treatment of chronic lymphatic leukemia (CLL) in 1952 and since then it has been the most common treatment for CLL<sup>1</sup>. It is also an immunosuppressive agent that has been used to treat systemic lupus erythematosus, acute and chronic glomerular nephritis, nephrotic syndrome, psoriasis, Wegener's granulomatosis, chronic active hepatitis, and cold agglutinic disease<sup>2,3</sup>. Chlorambucil (CBC) has been used in the treatment of a wide spectrum of malignancies such as lymphocytic leukemia, lymphoma, giant follicular lymphoma, chronic lymphocytic lymphoma, Hodgkin's disease, lymphosarcoma, breast cancer, and others<sup>4,5</sup>. The anticancer effect of CBC has been attributed to its binding ability with DNA, RNA and cellular proteins<sup>6</sup>. Reaction of one of the two-chloroethyl groups of chlorambucil (Fig. 1a) with the  $N^7$  position of guanine or adenine of double-stranded DNA leads to the formation of

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mono-adducts. These are repaired rapidly in an DNA repair error-free fashion by protein O6-methylguanine DNA methyltransferase (MGMT). However, some cells lack this repair activity, usually because of silencing of the corresponding gene, and the unrepaired DNA mono-adduct then forms a complex with mismatch-repair enzymes. The subsequent inhibition of DNA replication can eventually induce DNA breakage7. The second chloroethyl group of the DNA mono-adduct with chlorambucil can interact with proteins<sup>8</sup>.

The CBC induced major side effects include myelosuppression, pancytopenia, anaemia,



Fig. 1—Chemical structure of chlorambucil (a) and ascorbic acid (b).

thrombocytopaenia and/or leukopaenia, renal toxicity<sup>5,9,10</sup>, gastrointestinal toxicity, fertility disorders<sup>9</sup> and neurotoxicity<sup>11-13</sup>. Hepatotoxicity and pneumotoxicity have been reported only infrequently  $^{9,10}$ . In the endeavor to decrease drug-induced toxicity in the host without decreasing the therapeutic efficacy, the use of anticancer drugs such as cisplatin<sup>11</sup>, cyclophosphamide<sup>12</sup> , paclitaxel<sup>13</sup> and arsenic trioxide<sup>14</sup> in combination with vitamin C (2-Oxo-Lthreo-hexono-1,4-lactone-2,3-enediol) have been examined.

The name vitamin C (Fig. 1b) refers to the L-enantiomer of ascorbic acid and its oxidized form. The opposite D-enantiomer called D-ascorbate has equal antioxidant power, but is not found in nature, and has no physiological significance. Ascorbic acid (L, 3-ketothreohexuronic acid lactone), is the most important water-soluble biological antioxidant which can scavenge both reactive oxygen species and reactive nitrogen species. The role of ascorbic acid in medicine in general and in anticancer therapy has been discussed controversially<sup>15</sup>. Cameron and Pauling<sup>16</sup> found four-fold increase in the survival time of ascorbic acid supplemented patients having terminal human cancers. While many other studies have reported good therapeutic potential of vitamin C against cancers<sup>17-19</sup>, some have shown virtually no benefit from vitamin C treatment<sup>20</sup>. Role of ascorbic acid has also been suggested in inhibiting carcinogenesis<sup>21-23</sup> or enhancing carcinogenesis<sup>24,25</sup>. Some genotoxic effects of vitamin C *in vitro* test systems has been demonstrated<sup>26,27</sup> 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<sup>28</sup>. It plays an important role in the protection of cells against various types of oxidant injury, for example, TNFαinduced apoptosis in micro-vascular endothelial cells<sup>29</sup> and in human umbilical vascular endothelial cells<sup>30</sup>, hypoxia-reoxygenation-induced apoptosis<sup>31</sup> and against  $\beta$ -amyloid-induced cell death and apoptosis in neuroblastoma SH-SY5Y cells<sup>32</sup>. Further, ascorbic acid exerts a protective effect against oxidized LDL induced toxicity in THP-1-derived macrophages<sup>33</sup>. The protective role of ascorbic acid against cisplatin-induced mutagenic and nephrotoxic effects has also been noted with possible cooperative involvement of GSH in its protective function<sup>34</sup>.

Thus, considering the variable findings on the significance of vitamin C in relation to cancer

chemotherapy and possibility of development of CBC-induced toxicity in mice, the present study has been undertaken to assess the modulatory effect of dietary ascorbic acid on CBC-mediated antitumor efficacy and hematological and renal toxicity in mice with experimental malignant murine tumor which has the significance in cancer treatment in general.

# **Materials and Methods**

*Chemicals*—Chlorambucil, heparin, osmium tetroxide, cacodylate buffer, acridine orange, ethidium bromide, giemsa and trypan blue were purchased from Sigma Chemical Company St. Louis, U.S.A. Ascorbic acid, WBC and RBC diluting fluid, ethanol and other chemicals used in the experiments were of analytical grade and purchased from SRL Pvt. Ltd., Mumbai, India.

Animals and tumor maintenance—Swiss albino mice were maintained under conventional laboratory conditions at  $24 \pm 2$  °C with free access to food pellets (Amrut Laboratory, Delhi, India) and water *ad libitum*. Ascites Dalton's lymphoma (DL) tumor was maintained *in vivo* by serial intraperitoneal (ip) transplantation of viable tumor cells to animals (in 0.25 mL PBS, *p*H 7.4). The tumor-transplanted animals usually survived for about 19–21 days.

Dose standardization—Chlorambucil was initially dissolved in 70% ethanol and subsequently diluted with PBS (phosphate buffer saline) as described by Russell *et al*<sup>35</sup>. Intraperitonial injections were made immediately after dissolving the chemical and were always completed within 30 min (keeping the solution on ice in the interim) and their anticancer activity was determined by following the method described by Ahluwalia et al<sup>36</sup>. Tumor-bearing hosts were treated with different doses (6,8,10,12,14,16, 18 and 20 mg/kg body weight) of chlorambucil on the 8<sup>th</sup> day of tumor transplantation and finally, the dose of 10 mg/kg body weight was selected based on better anticancer activity, which is the half of  $LD_{50}$  dose. Dose of ascorbic acid was selected based on our earlier findings<sup>11,12,37</sup> and the same showed better result in the present studies also.

Antitumor study—Antitumor activity of chlorambucil and ascorbic acid alone or in combination was studied following the method of Sakagami *et al.*<sup>38</sup>. Viable DL tumor cells  $(1 \times 10^7)$  were transplanted intraperitoneally in 10-12 weeks old male mice (30 g body wt.). The day of tumor

transplantation was designated as day zero. For antitumor study, tumor-transplanted mice were randomly divided into following 4 groups of 10 mice each Group- I, II, III and IV served as control, chlorambucil treatment, ascorbic acid treatment and ascorbic acid plus chlorambucil treatment respectively and the details of treatment schedule are shown in Fig. 2. Survival patterns of the hosts in each group were monitored and recorded. Antitumor efficacy was expressed in percentage of average increase in life span (ILS) calculated using the formula  $(T/C \times 100)$ -100, where, T and C are the mean survival days of treated and control groups of mice, respectively. For the apoptosis and cell cytotoxicity studies, in separate experiments, mice in different groups were killed by cervical dislocation and DL cells were collected at 24, 48, 72 and 96 h intervals post treatment. The maintenance, use of the animals and the study protocol of the present experiments have been approved by the Institutional Ethical Committee, North-Eastern Hill University, Shillong.

Cell viability study using Trypan blue exclusion test-Cell viability was checked by trypan blue exclusion test as described by Talwar *et al*<sup>39</sup>. To analyze the comparative cytotoxicity of the chlorambucil (CBC) and ascorbic acid (AA) alone and in combination, the DL cells were collected from mice at different duration (24, 48, 72 and 96 h) and washed twice with PBS. Aliquot of the cell suspension was mixed with an equal volume of trypan blue (0.4% in PBS) and incubated for 10 min. Number of viable and dead cells were determined with Neubauerhaemocytometer under а light microscope. The percentage of viability was calculated using the formula:



Fig. 2—Schedule of drug treatment in tumor bearing mice and tumor collection at different time point for experiments.

Viability (%) = 
$$\frac{\text{total viable cells of treated}}{\text{total viable cells of control}} \times 100$$

Apoptosis study using fluorescence microscope— Fluorescence based apoptosis was determined in DL cells using acridine orange and ethidium bromide (AO/Etbr) staining method<sup>40</sup> previously used by Verma and Prasad<sup>41</sup>. After treatment, the DL cells were collected from mice at different time intervals (24, 48, 72 and 96 h). The cells were washed twice with PBS and stained with AO/Etbr (100 µg/mL PBS of each dye) for 5 min and again washed. The cells from different treatment groups were observed and thoroughly examined under fluorescent microscope and photographed. The viable cells nuclei stain green due to permeability of only acridine orange whereas, apoptotic cells appear red due to co-staining of both the stains. One thousand cells were analyzed and percentages of apoptotic cells were counted from twenty selected view fields under microscope.

Scanning Electron Microscopy (SEM)—The DL cells pellet collected from the animals under varying experimental conditions were used for scanning electron microscopy. The DL ascites collected from the peritoneal cavity was centrifuged (1000 g for 10 min at 4° C). The cells pellet was washed once in PBS and the cell pellet was resuspended in PBS (1:4, w/v) to get cell suspension which wasthen fixed in 2.5% (v/v) glutaraldehyde at 4 °C. Fixed cells were rinsed in 0.1 M phosphate buffer, pH 7.2 and post-fixed with 1% osmium tetroxide. Cells were rinsed in phosphate buffer and dehydrated in a graded ethanol series of 30, 50, 70, 90 and 100% for 20 min each. Cells were then critical point-dried in a critical point dryer (CPD-030, BAL-TEC Co.) and were affixed to an aluminum stub with double-stick tape, coated with gold in an ionic sputter coater (SCD-005, BAL-TEC Co.). They were viewed, examined thoroughly and photographed under the scanning electron microscope (JEOL JSM - 6360).

Kidney histopathology—Mice from different groups as described in Materials and Methods were killed after 15 days of the treatment and kidneys were collected for histopathological studies as described by Yang *et al*<sup>42</sup>. Slices of the left kidney (from five animals in each group) were fixed in 10% formalin for 48 h and were embedded in paraffin. Thin sections (8-10 µm thickness) were cut and collected on glass slides, deparaffinized and stained with hematoxylin and eosin stain. The stained sections were examined under a light microscope (Leica DFC425 C) and the cellular features and any deformities were recorded. *Hematological studies*—Blood samples from the mice in different groups were collected from the tail vein into a sterilized tube containing heparin (15-20 IU per mL of blood) and used for the hematological analysis. RBC count, WBC count and haemoglobin content in blood were determined as per Dacie and Lewis<sup>43</sup>.

Analysis of morphological alterations in RBCs— The blood collected from the eyes of the mice under varying experimental conditions was gently smeared on the cover slips and fixed in 2.5% (v/v) glutaraldehyde at 4 °C for SEM analysis. Fixed cells were further processed as mentioned above in case of DL cells for scanning electron microscopy.

Statistical analysis—All values in the present study indicate mean±SD, and all determinations were repeated thrice. One-way analysis of variance (Tukey), and the independent sample *t*-test was used to know the significance of differences between two treatment groups. *P* values  $\leq 0.05$  were considered as statistically significant.

# Results

The percent survival patterns of tumor-bearing mice in different experimental groups have been shown in Fig. 3. Out of the different sub-lethal doses of CBC screened for antitumor activity, 10 mg/kg body weight dose was found to be the most effective against murine ascites Dalton's lymphoma (Table 1). The increase in life span at the dose 10 mg/kg was dose found to be 140%, whereas, in case of combined treatment with AA and CBC (10 mg/kg body wt.) the ILS was further increased to 170%. A significant time dependent increase ( $P \le 0.05$ ) in the percentage of cell



Fig. 3—Graph showing the survival pattern of tumor-bearing mice in control and different treatment groups.

cytotoxicity was observed after the cells were treated with CBC or AA plus CBC (Fig. 4). Combination treatment showed more tumor cells cytotoxic effect as compared to CBC alone treatment.

Acridine orange is a vital dye that stains both live and dead cells, whereas, ethidium bromide stains only those cells that have lost their membrane integrity<sup>40</sup>. Cells stained green represent viable cells, whereas orange/red stained cells represent apoptotic cells. The percentage of apoptotic cells at different treatment duration is shown in Fig. 5 and represents the apoptotic and viable cells of combined treatment group only. Control DL cells were rounded in shape with uniform green fluorescence while CBC treatment after 96 h caused nuclei constriction and early membrane damage (Fig. 5 a and b). AA treatment after 96 h showed reduction in cell volume with very less apoptotic cell death while AA plus CBC

Table 1—Antitumor activity of chlorambucil (CBC) at different doses used during dose standardization in tumor-bearing mice [Day of treatment=8th day; no. of mice in each group=10]

Treatment dose (mg/kg, ip)	ILS (%)		
Control	_		
6	75		
8	80		
10	140		
12	120		
14	100		

Each set of experiments was repeated thrice (n=3) and observed almost similar result.



Fig. 4—Analysis of dead DL cells (cytotoxicity) from mice treated with ascorbic acid, chlorambucil and ascorbic acid plus chlorambucil determined by trypan blue exclusion test. Results are expressed as mean  $\pm$  SD, n=3, \*P $\leq$ 0.05 as compared to chlorambucil alone treatment.

treatment for 24-96 h showed cell shrinkage, and loss of cell membrane integrity and appearance of membrane blebbing (Fig. 5 c and d). After 72-96h (Fig. 5 f and g) of incubation period severe changes in cellular morphology, including chromatin condensation, membrane blebbing, and numerous fragmented nuclei were observed. Large size cytoplasmic and membrane vacuoles were also noticed with loss in membrane integrity. There was a significant ( $P \le 0.05$ ) increase in percent apoptotic cells in combined treatment condition as compared to CBC alone treatment (Fig. 6).

To further examine and ascertain the apoptotic features and the surface morphological changes in DL cells, SEM studies were also undertaken (Fig. 7). The control untreated cells showed numerous microvilli on the surface of the cells and ruffles distributing evenly on the cell surface (Fig. 7a). AA treatment for 96 h showed shrinkage and abnormal appearance of plasma membrane with complete disappearance of microvilli on the cell surface (Fig. 7b). CBC treatment for 96 h (Fig. 7c) exhibited a decrease in the number of microvilli and an appearance of membrane blebbing with cell membrane deformities. The surface of DL cells appeared relatively smooth with no obvious microvilli after 24 h of combination treatment

(Fig. 7d). Severe cell membrane folding and cell shrinkage were observed after 48-96 h of AA plus CBC treatment (Fig. 7 e-g). Some phagocytic cells were also visible in association with the tumor cells (Fig. 7 d-e, dotted arrow).

Histopathological examination of kidney from the mice in control group showed normal renal



Fig. 6—Percent apoptotic cells or apoptotic index in DL cells of mice at different treatment conditions. Results are expressed as mean  $\pm$  SD, n=3, \* $P \leq 0.05$  as compared to chlorambucil alone treatment.



Fig. 5—Morphological observation of viable and apoptotic DL cells after acridine orange and ethidium bromide (AO/EB) staining. (a) Control DL cells treated with drug vehicle alone; (b) Chlorambucil treatment after 96 h; (c) Ascorbic acid treatment after 96 h; (d-g) Ascorbic acid plus chlorambucil treatment for 24-96 h. During 24-48 h of combination treatment membrane blebbing, nucleus shrinkage and chromatin condensation were noticed while at later periods of treatment (72-96 h) showed fragmented nuclei, apoptotic bodies and complete loss in plasma membrane integrity. Viable DL cells stain green whereas, apoptotic cells stain red/orange.

architecture of both outer cortex and inner medulla. Outer cortex showed a normal structure of renal glomeruli, surrounded by a double-walled epithelial capsule called Bowman's capsule (Fig. 8a and b). The proximal convoluted tubules were noticed to be lined with typical thick cubic epithelium. The distal convoluted tubules show considerably lower cubic epithelium (Fig. 8c). A medullary region consists of collecting tubules lined with the relatively low simple cubic epithelium. The thick descending and ascending parts of Henle's loops are lined with simple cubical epithelium with small caliber, and a small amount of interstitial tissue can be seen normally in the cross sections (Fig. 8c). The major histopathological abnormalities observed after CBC treatment (Fig. 8d-f) were vacuolization in tubular cells, many areas of interstitial mononuclear cell tubular damages, infiltration, focal necrosis and haemorrhage. Magnified view of glomerulus (Fig. 8e) showed some vascular glomeruli which were apparently enlarged, tightly filling the Bowman's capsule with absence of the capsular spaces. AA plus CBC treatment (Fig. 8g-i) showed reduction in these histopathological toxic effects in kidney. There were less interstitial mononuclear cell infiltration with minimal vacuolization and congestion (Fig. 8i) as compared with CBC treatment. No

abnormality was observed in the experimental group treated with AA alone.

Analysis of various hematological parameters used for CBC mediated hematotoxicity studies in tumor bearing mice indicated that the RBC and WBC counts and hemoglobin content decreased after CBC treatment as compared with control mice. AA alone treatment showed similar pattern resembling that of control group. However, combination treatment of mice with AA plus CBC resulted in an expected rise and recovery in all hematological parameters (Table 2).

Scanning electron microscopic study of RBCs morphology showed a significant increase in CBCinduced morphological abnormalities in RBCs (erythrocytes). The major abnormalities scored after CBC treatments were acanthocytes, microcystis, scalloping, echinocytes, elliptocytes and burr cells (Fig. 9). The number of normocytes decreased significantly after CBC treatment (Fig. 9b). Control and AA treatment showed the similar pattern in RBC morphology (Fig. 9a-c). Combination treatment with AA plus CBC resulted in a significant ( $P \le 0.05$ ) decrease in abnormal RBCs as compared to CBC alone (Figs 9 and 10). Various morphological features observed in RBCs were as control erythrocytes or normocytes with smooth surface and biconcave shape,



Fig. 7—Scanning electron micrograph of DL cells. (a) Control group showed rounded shape with few membrane projections and ruffles distributed evenly over the cell surface; (b) ascorbic acid treatment (96 h); (c) chlorambucil treatment (96 h). Ascorbic acid plus chlorambucil treatment after 24 h (d); 48 h (e); 72 h (f) and 96 h (g). Dotted arrow shows leukocytes infiltration towards DL cells and regular arrow indicates important features on the cells surface such as membrane blebs and cell membrane deformities (f, g).



Fig. 8—Histological features of kidney from control and treatment group after 15<sup>th</sup> day. First lane showing renal tubules and glomerulus in control and treated mice, second lane showing glomerulus under higher magnification and third lane showing renal tubule in higher magnification. Kidney histology of control group (a-c), chlorambucil treatment (d-f), and combination treatment (g-i). There was no significant changes in histological pattern in AA- treated group.

microcytes having lesser diameter than the normocytes, acanthocytes with few horn like projections over the surface, scalloping types with folded membrane, elliptocytes cells depicting elliptical shape, echinocytes with serrated-projections distributed evenly over the cell surface and burr cells with spiny projections from the cell surface.

# Discussion

In the studies related with the assessment of antitumor activity of various drugs, ascites Dalton's lymphoma has been commonly used as an important murine experimental tumor model<sup>11,12,37,44</sup>. As this malignant Dalton's Lymphoma is related with the lymphocytes (mainly T-lymphocytes), it may also be helpful in correlating some human cancer related with lymphocytes such as precursor T-cell leukemia/lymphoma, non-Hodgkin lymphoma, Burkitt's lymphoma, BCL, Follicular lymphoma, MALT lymphoma etc. Chlorambucil and its derivates are alkylating agents which have been used against various malignancies particularly chronic lymphocytic leukemia (CLL)<sup>5,6</sup>. Chlorambucil has also been used combination with other agents in such as

[Results are mean±SD from 3 observations erach]								
					Treatment			
Parameters	Normal	Time (h)	DL control	CBC	AA	CBC+AA		
RBC (x10 <sup>12</sup> /L)	7.69±0.60	24	6.69±0.70	5.76±0.25*	6.55±0.42	6.58±0.28		
		48	6.42±0.48	5.10±0.49*	6.40±0.20 <sup>#</sup>	6.45±0.32 <sup>#</sup>		
		72	6.25±0.37	4.16±0.32*	6.18±0.35 <sup>#</sup>	6.20±0.25 <sup>#</sup>		
		96	6.10±0.22	3.29±0.65*	6.12±0.55 <sup>#</sup>	6.15±0.46 <sup>#</sup>		
Hb(g/dL)	14.90±0.65	24	13.05±0.68	11.90±0.54*	12.94±0.56 <sup>#</sup>	12.09±0.54		
		48	12.78±0.74	11.20±0.63*	12.67±0.63 <sup>#</sup>	12.02±0.37		
		72	12.61±0.58	10.85±0.66*	12.50±0.72 <sup>#</sup>	$11.77\pm0.46^{\dagger}$		
		96	12.46±0.63	10.07±0.61*	12.35±0.75 <sup>#</sup>	$11.69 \pm 0.33^{\ddagger}$		
WBC (x10 <sup>9</sup> /L)	5.14 ±0.49	24	8.39±0.27	4.93±0.82*	8.02±23 <sup>#</sup>	5.42±37		
		48	10.87±0.29	5.42±0.63*	10.11±45 <sup>#</sup>	$6.84\pm55^{\#}$		
		72	11.21±0.78	5.93±0.33*	11.32±30 <sup>#</sup>	$6.99 \pm 23^{\#}$		
		96	12.49±0.84	6.52±0.41*	12.56±62 <sup>#</sup>	$7.82\pm69^{\#}$		

 Table 2—Changes in the hematological parameters in tumor-bearing mice under different treatment condition
 [Results are mean±SD from 3 observations erach]

ANOVA,  $P \leq 0.05$ : \*as compared to the corresponding control and <sup>#</sup>as compared to chlorambucil alone treatment at corresponding time of treatment. Normal = Blood from the mice without tumor or treatment; Control=Blood from untreated tumor bearing hosts receiving drug vehicle alone; AA=Ascorbic acid, CBC=Chlorambucil; RBC=Red blood cells, WBC=white blood curpuscles and Hb=Haemoglobin.

fluderabine<sup>45</sup>, 2-(morpholin-4-yl)-benzo[h]chomen-4-one<sup>46</sup>, levamisole<sup>47</sup> against various cancers. The host survival data from the present studies indicate significant increase in survivability of the tumorbearing mice treated with AA plus CBC (Fig. 3), as compared to the group of mice treated with either suggesting additive/ synergistic agent alone. antitumor activity of AA and CBC against murine Dalton's lymphoma. The analysis of cell viability of DL cells under different treatment conditions revealed that the number of dead DL cells was increased significantly in mice treated with AA plus CBC as compared to either treatment alone. This clearly shows the increased cytotoxicity of DL cells under combined treatment condition (Fig. 4). Ascorbic acid at a nontoxic concentration, in combination with certain other pharmacological agents has also been reported to produce an enhanced cancer growth inhibition effects, such as pharmacological doses of ascorbic acid enhanced the effects of arsenic trioxide on ovarian cancer cells<sup>48</sup> gemcitabine on pancreatic cancer cells<sup>49</sup> and combination treatment of gemcitabine and epigallocatechin-3-gallate (EGCG) on mesothelioma cells<sup>50</sup>. The combination treatment with ascorbic acid and hydrogen peroxide caused a significant decrease in the glutathione levels in the murine neuroblastoma cells and it may lead to

effective death of cancer cells<sup>51</sup>. It has also been reported that high-dose ascorbate increases radiosensitivity of glioblastoma multiforme cells, resulting in more cell death than from radiation alone<sup>52</sup>. Uncontrolled proliferation and a defect in apoptosis regulating pathways represent crucial elements in the development and progression of malignant tumors<sup>53</sup>. Many chemotherapeutic drugs including chlorambucil have been reported to induce cytotoxic effects against cancer cells mainly through programmed cell death or apoptosis<sup>54,55</sup>. Apoptosis is characterized by membrane blebbing, shrinking of cells and their organelles, DNA fragmentation, and finally cell disintegration<sup>56</sup>. The assay based on AO/EtBr fluorescence staining is a good reliable analysis for the authentication of apoptotic features as compared to other methods<sup>57</sup>. The results of present AO/EtBr based apoptotic related analysis showed higher apoptotic index in DL cells after combination treatment with ascorbic acid and chlorambucil as compared to chlorambucil alone (Figs 5 and 6) which also supports the observed enhanced cytotoxicity in DL cells after combination treatment (Fig. 4). These findings are in conformity with the earlier report showing better therapeutic efficacy and higher apoptotic index in DL cells after the combination treatment with AA plus cisplatin as compared to



Fig. 9—Scanning electron micrograph RBCs in control and different treatment groups. (a) control; (b) chlorambucil treatment after 96 h; (c) ascorbic acid treatment after 96 h; combination treatment with ascorbic acid plus chlorambucil for 24 h (d), 48 h (e), 72 h (f) and 96 h (g) Lower panel showing the different major abnormal shaped RBC observed after treatment.

cisplatin alone<sup>11</sup>. The use of chlorambucil with MDM2 antagonists such as nutlins has been found to elevate p53 by inhibition of interaction of the negative regulator MDM2 with p53 and that induces p53-dependent apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells<sup>58, 59</sup>.

Several mechanisms have been elucidated to explain the AA-mediated enhancement in the antitumor activity and apoptosis of anticancer drugs. There is also increasing evidence that AA is selectively cytotoxic to some types of tumor cells, functioning as a pro-oxidant, rather than anti-oxidant. It has been reported that AA induces apoptosis with the generation of GSH oxidation and  $H_2O_2$ accumulation in acute myeloid leukemia (AML) cells. Induction of apoptosis in AA-treated AML cells involved a dose-dependent increase of Bax protein, release of cytochrome C from mitochondria to cytosol, activation of caspase 9 and caspase 3, and cleavage of poly (ADP-ribose) polymerase<sup>60</sup>. The pro-oxidant activity of ascorbic acid is due to its ability to redox-cycle with transition metal ions, and



Fig. 10—Histogram showing the percentage of normal and abnormal shaped RBC in control and different treatments groups. Results are expressed as mean  $\pm$  SD, n=3, \**P*<0.05 as compared to control and <sup>#</sup>*P* $\leq$ 0.05 as compared to the chlorambucil alone treatment.

thereby stimulates the formation of species such as superoxide, hydrogen peroxide and hydroxyl radicals. *In vitro* studies have shown that ascorbic acid in high

concentrations enhances the cytotoxicity of 5fluorouracil in a dose-dependent manner in mouse lymphoma<sup>61</sup>. Sodium ascorbate-mediated apoptosis has also been suggested to be initiated by a reduction in transferrin receptors expression and decrease in iron uptake into tumor cells, which is necessary for maintenance of tumor cells proliferation<sup>62</sup>. It has been reported that ascorbic acid increases the apoptosis via up-regulation of p53 during cisplatin treatment of human colon cancer cells<sup>63</sup>. In ascorbatesupplemented 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<sup>64</sup>. Vitamin C (10 mM) induces apoptosis in B16 murine melanoma cells by decreasing mitochondrial membrane potential and release of cytochrome  $c^{65}$ . Kim *et al*<sup>66</sup>. suggested that vitamin C induces apoptosis of colon cancer cells through the increase of (i) the calcium influx in endoplasmic reticulum (ER), (ii) the translocation of Bad to mitochondria, and (iii) the expression of Bax. These variable anticancer effects involving various mechanisms related with AA induced apoptosis may not be universally applicable against all the cancers. This differential sensitivity could be partly associated with differences in AA uptake by different cancer cells. SEM observations revealed a series of cytological changes during DL regression following CBC and combination treatment with AA plus CBC. Treatment of DL cells with AA plus CBC (Fig. 7) resulted in significant alterations in morphological features which included complete loss of fine membrane projection, cell shrinkage and formation of large size membrane blebs without ruffles throughout cell membrane. The interaction of tumor cells with the phagocytic cells through appearance of fine membrane projections after AA plus CBC treatment has also been found (Fig. 7). This infiltration of leukocytes towards cancer cells also reveals the disintegration in the plasma membrane of tumor cells surrounded/connected by leukocytes which could be due to the release of some toxic factors from the leucocytes. AA plus CBC treatment of DL cells for 72-96 h resulted in significant morphological alterations, with a complete loss of fine membrane projection, cell shrinkage and formation of large size membrane bleb without ruffles throughout cell

membrane which are typical characteristics of apoptotic cell death which corroborate the apoptosis related findings by AO/Etbr staining methods.

The major histopathologic abnormalities in kidney (Fig. 8) observed after CBC treatment were vacuolization in tubular cells, tubular damages, interstitial mononuclear cell infiltration, focal necrosis and haemorrhage. Glomerulus at higher magnification showed some enlarged vascular glomeruli, tightly filling the Bowman's capsule with absence of the capsular spaces. Damage to the epithelium of proximal tubules, in the most severe cases of distal tubules, and haemostasis and bleeding in the medullar and cortical area has been found to be associated with CBC induced renal toxicity<sup>67,68</sup>. Combination treatment of mice with AA plus CBC showed reduction in these CBC-induced nephrotoxic effects (Fig. 8 g-i) as there were less interstitial mononuclear cell infiltration with minimal vacuolization and congestion (Fig. 8i) as compared with CBC treatment. This finding on the protective role of AA against cancer chemotherapeutic drugs-induced nephrotoxicity is supported from the earlier study by Weiil *et al.*<sup>69</sup> in which cancer patients received cisplatin-based chemotherapy, and half the patients were given a dietary supplement that consisted of vitamin C, vitamin E and selenium. The patients receiving vitamin C showed recovery with respect to the severity of the nephrotoxicity induced by cisplatin. In another study<sup>70</sup> using combination of cisplatin with antioxidants such as vitamin C against murine visceral leishmaniasis also resulted in successful reduction of nephrotoxicity by normalizing the enzymatic levels of kidney function tests, along with the reduction in parasite load and increase in Th1 type of immune responses.

Development of CBC-induced blood related toxicity such as myelosuppression- pancytopenia, anaemia, thrombocytopaenia and/or leukopaenia<sup>5,9,10</sup> is another problem. Depletion in erythrocytes leads to iron deficiency, anemia<sup>71</sup> and is a frequent complication of cancer diseases. Tung *et al.*<sup>72</sup> mentioned that the reduction in the values of blood parameters like RBC, WBC and Hb may be attributed to the hyperactivity of bone marrow, which leads to production of red blood cells with impaired integrity that are easily destroyed in the circulation. In the present study also a significant decrease in the haematological parameters i.e. RBC, WBC counts and Hb contents, were observed in tumor-bearing

animals after CBC treatment (Table 1). AA plus CBC co-treatment of tumor-bearing animals caused significant recovery in these haematological values (Table 2). Scanning electron microscopic study also revealed CBC- induced morphological abnormalities in erythrocytes. The major abnormalities observed after CBC treatments are acanthocytes, microcystis, scalloping, echinocytes, elliptocytes and burr cells (Fig. 9). The number of normocytes decreased significantly after CBC treatment (Fig. 9b). Combination treatment with AA plus CBC resulted in a significant ( $P \le 0.05$ ) decrease and betterment in abnormal RBCs as compared to CBC alone (Figs 9 and 10).

The decreased life span of RBCs and anemia may be correlated with decreased blood antioxidant capacity<sup>73</sup>. In addition to oxygen transport, RBCs also function as conveyors of nutrients, and serve as targets for drugs, pathological factors and environmental xenobiotics<sup>74</sup>. Severe CBC mediated hematotoxicity and bone marrow suppression are also reported by many workers<sup>1,5,75</sup>. Ascorbate (vitamin C) is considered to be an important antioxidant in extracellular fluid including blood and protects plasma lipids from peroxyl radicals mediated peroxidative damage $^{76}$ . Ascorbic acid has been reported to cause protective effect against hematological toxicity induced by chlorpyrifos<sup>77</sup> and carbamazepine<sup>78</sup> also in rats. The vitamins (C and E), supplementation has also been found to be associated with reduced toxic effects of ethanol on liver weight and some blood parameters in rabbit<sup>79</sup>.

In conclusion, it may be suggested that combination treatment of AA plus CBC could be very useful in enhancing CBC-mediated therapeutic efficacy which involves induction of apoptosis in DL cells with higher apoptotic index. CBC treatment induced hematotoxicity and renal toxicity in the host but the treatment with AA plus CBC showed significant decrease in these toxicities indicating a protective effect, thus, indicating differential effects of the combined treatment on the cancer cells and other tissues of the host.

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# **Conflict of interest**

We do not have any conflict of interest for the present paper.

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