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MODULATORY EFFECT OF ASCORBIC ACID (VITAMIN C) ON CYCLOPHOSPHAMIDE-MEDIATED ANTITUMOR ACTIVITY AND MUTAGENICITY IN MICE BEARING ASCITES DALTON'S LYMPHOMA

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ABSTRACT

Cyclophosphamide is an anticancer drug with immunosuppressive activities. Considering various reports on the possible antioxidant/protective functions of ascorbic acid, it is aimed at to explore the modulatory effect of ascorbic acid on therapeutic efficacy as well as mutagenic potentials of cyclophosphamide in the same tumor bearing mice. Swiss albino mice with ascites Dalton's lymphoma were used for the experiments. Combination treatment of mice with ascorbic acid and cyclophosphamide showed a significant increase in life span as compared to that of cyclophosphamide alone. As compared to cyclophosphamide alone, the frequency of mutagenic parameters were lessened after combination treatment with ascorbic acid and cyclophosphamide. The glutathione level in tumor cells increased with tumor growth. The treatment of tumor-bearing mice with cyclophosphamide alone or in combination with ascorbic acid caused a decrease in protein, glutathione levels and lactate dehydrogenase (LDH) activity, and an increase in lipid peroxidation (LPO) in tumor cells.

KEYWORDS

Cyclophosphamide, ascorbic acid, antitumor activity, mutagenicity, glutathione, lipid peroxidation

INTRODUCTION

Alkylating agents have the ability to add alkyl groups to many electronegative groups under conditions present in cells and have proved to be quite useful in cancer chemotherapy. Cyclophosphamide (*N*,*N*-bis[2-chloroethyl]-1,3,2-oxazaphosphinan-2-amine 2-oxide; brand name cytoxan), is an anticancer alkylating chemotherapeutic drug[1-3] with immunosuppressive activities[4]. Cyclophosphamide (CP) has been widely used in the treatment of a wide spectrum of malignancies such as leukemia, lymphoma and the cancer of breast, lung, prostrate, ovary[1,5,6] and also in autoimmune diseases[7,8] and organ transplantation[9-11]. The parent compound is inactive *in vitro* and *in vivo*. It is a prodrug that is activated by cytochrome P450 enzymes predominantly in liver to generate active alkylating metabolites such as phosphoramide mustard and acrolein[12-14] which crosslink DNA between (interstrand cross-linkages) and within (intrastrand cross-linkages) DNA strands at guanine

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N-7 positions. This may cause prevention of cell division. It is considered to be cell cycle phase-nonspecific[14]. The active metabolites of cyclophosphamide circulate through the blood and gains entry to tumor tissue to exert its therapeutic effects. However, it can also reach to other sensitive organs and normal cells to cause toxicities, thus, limiting its full therapeutic efficacy. Long term use of cyclophosphamide has been reported to develop multiple side effects which may include nausea and vomiting, bone marrow suppression, hemorrhagic cystitis, urotoxicity, darkening of the skin, alopecia (hair loss) or thinning of hair, infertility etc[15-17]. It may also be associated with a risk of developing secondary malignancies[18-20]. The active metabolites of cyclophosphamide may also cause genotoxic effects by inducing chromosomal aberrations and development of micronuclei in the normal cells of the host[21-23].

Ascorbic acid (L,3-ketothreohexuronic acid lactone), commonly known as Vitamin C, is one of the most important reducing agents occurring in living tissue. While most animals synthesize their own vitamin C, humans and a few other animals, such as non-human primates, guinea pigs, and fruit bats do not. It is an active reducing agent involved in numerous biological effects and detoxification of many endogenous and exogenous compounds [24,25]. It is necessary in the body to form collagen in bones, cartilage, muscle, and blood vessels, and aids in the absorption of iron[26-27]. Chemo-preventive and therapeutic role of vitamin C against cancers have been widely reported [28,29]. The various reports on the protective function of dietary ascorbic acid against some anticancer agents-induced clastogenic effects [30-34] and tissue toxicities [35-39] have drawn increasing attention. 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[40, 41]. While many studies have reported the good therapeutic potential of vitamin C against cancer [40, 42-45] others have shown virtually no benefit from vitamin C treatment[46]. Some conflicting role of ascorbic acid has also been suggested in either inhibiting [47-50] or enhancing carcinogenesis in some mammalian models like guinea pig, rats [51,52] and mice[53]. Some genotoxic effects of vitamin C *in vitro* test systems has been demonstrated[54,55], but in the experiments *in vivo*, there are no genotoxic effects by vitamin C.

Thus, considering these variable findings on the significance of vitamin C in relation to cancer chemotherapy and possibility of development of drug-induced mutagenicity, it became a topic of interest to evaluate the effectiveness of ascorbic acid on cyclophosphamide-induced antitumor activity and mutagenicity in the same tumor-bearing mice. This may give us better understanding on the correlation of these two modulatory effects, because, most of the studies related with the use of vitamin C in combination with anticancer drugs for elucidating any change in therapeutic efficacy or drug-induced mutagenic potentials has been done separately with different experimental set up/animals.

The average changes in the hosts survivality, protein, glutathione, lipid peroxidation (LPO), and lactate dehydrogenase (LDH) activity in tumor cells of mice under different treatment conditions were determined. The development of chromosomal aberrations and micronuclei in the cells has been well established sensitive biological indicator of scrutiny of drug-induced mutagenicity[30-33, 56] so, these parameters were studied in bone marrow cells of the mice under the same experimental conditions as was used for antitumor activity.

MATERIALS AND METHODS

Chemicals

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Cyclophosphamide, L-ascorbic acid (vitamin C), fetal calf serum were purchased from Sigma Chemical Co., USA. Giemsa stain and other chemicals of analytical grade were purchased from SRL Co., Mumbai, India. Various solutions were always prepared in double glass-distilled water.

Animals and Tumor maintenance

Inbred Swiss albino mice colony is being maintained in the laboratory under conventional conditions with commercially available food pellets (Amrut Laboratory animal feeds, New Delhi) and water *ad libitum.* Ascites Dalton's lymphoma is being maintained *in vivo* in 10 to 12 weeks old mice by serial intraperitoneal (i.p.) transplantation of 1 x 10^7 viable tumor cells (in 0.25 ml phosphate buffered saline, pH 7.4) per animal. Tumor-transplanted mice usually survived for 19-21 days. The maintenance and use of these animals for the experiments has been cleared by institutional ethical committee. Only the tumor-transplanted animals were used in present studies.

Drugs treatment schedule, hosts survival and antitumor activity

Tumor-transplanted mice of both sexes were randomly divided into four groups consisting of 10 mice each. Group-I mice served as control and received normal saline (0.89% NaCl) only. Group-II mice were given 1% ascorbic acid (AA) through drinking water from the 5th day of tumor transplantation. Based on the volume of water intake, the AA intake was noted to be about 15.8 – 17.3 mg/day/animal. Group-III mice were injected intraperitoneally (i.p.) with a single dose of CP (200 mg/kg body weight) on the 10th day post-tumor transplantation. The dose of CP was as per report by Czyzewska and Mazur[21] and according to the dose and treatment schedule used earlier by us[57]. Group-IV mice were given 1% ascorbic acid from the 5th day onwards and then administered i.p. with a single dose of CP on the 10th day of tumor transplantation. The survival patterns of the mice in different groups were determined and deaths, if any, were recorded daily. The anti-tumor efficacy of different treatments is reported as percent increase in life span and calculated as per the following formula:

(T/ C×100) – 100, where T and C are the mean survival days of treated and control mice, respectively.

The CP treatment schedule for 24, 48, 72 and 96 h has been used previously by Nicol and Prasad[57] and the same treatment schedule was followed here also. The average pH of the ascites tumor collected from mice in different groups was also determined. Ascites tumor was centrifuged (2000xg at 4°C, 10 min) and the pellet was used as DL cells and used for different determinations.

Glutathione estimation

Total glutathione (TGSH) contents were determined using the method of Sedlak and Lindsay[58]. Briefly, 5% homogenates of tissues were prepared in 0.02 mol/L EDTA, pH 4.7 in a motor-driven teflon–pestle homogenizer. TGSH was determined by adding 100µl of the homogenate to 1.0 ml of 0.2 mol/L Tris-EDTA buffer, pH 8.2 and 0.9 ml of 0.02 mol/L EDTA, pH 4.7 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 and the absorbency of supernatants were read against a reagent blank at 412 nm using a Beckman DU-640 spectrophotometer.

TGSH was also determined in the tumor cells every alternate day with tumor growth starting from the 4th day post tumor transplantation.

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Lipid peroxidation (LPO)

The lipid peroxidation (LPO) was determined as the concentration of thiobarbituric acid-reacting substances (TBRS), mainly malondialdehyde, in DL cells of mice in different groups using the method of Buege and Aust[59]. The tissue homogenate (5%) was prepared in 0.15 mol/L NaCl in a teflon–pestle tissue homogeniger. To 1 ml of homogenate, 2 ml of TCA (trichloroacetic acid)-TBA (thiobarbituric acid)-HCl reagent (15% TCA and 0.375% TBA dissolved in 0.25 mol/L HCl) was added and mixed thoroughly. The solution was heated in a boiling water bath for 15 min. After cooling at room temperature, the precipitate was removed by centrifugation (1000x*g*, 10 min). The absorbance of the supernatant was read at 535 nm. The malondialdehyde concentration in the sample was calculated using an extinction coefficient of 1.56 x 10^5 L/mol and is expressed as nmol/mg protein.

The protein contents were estimated in the same tissues as per the method of Lowry et al.,[60] using bovine serum albumin as a standard.

Lactate dehydrogenase (LDH)

Lactate dehydrogenase (LDH, EC 1.1.1.27, L-lactate:NAD oxidoreductase) activity in DL cells and corresponding ascites supernatant was determined according to the method of Cabaud and Wroblewski[61]. The molar extinction coefficient value of NADH at 340 nm is $6.22x10^6$. The unit of enzyme activity is expressed as the amount of enzyme which catalyzes the oxidation of one mole of NADH to NAD per minute at 30°C, and the specific activity of the enzyme was determined as the enzyme units per mg protein.

Chromosomal analysis

Mice from different experimental groups were subjected to mitotic arrest by administering colchicine (i.p., 4 mg/kg body wt), 1.5 h prior to sacrifice. Mice were killed by cervical dislocation and the bone marrow cells were collected from humerous and femur by flushing in warm (37°C) sodium citrate (1%) solution. Bone marrow cells were washed with Hank's balanced salt solution by centrifugation (1000xg, for 5 min at 4°C) and to the cell pellet, 9-10 ml of warm sodium citrate solution was added and incubated for 10-15 min. The cell suspensions were then centrifuged, and the cell pellet was fixed in acetic acid: methanol (1:3; v/v), repeated again with a 30 min interval. Before preparation of slides, fixed material was centrifuged and resuspended in a small volume of fixative and flushed gently until a cloudy suspension resulted. Two to three drops of this suspension were dropped on a clean slide previously chilled in 50% ethanol, burnt on a flame for a while, air-dried and stained on the following day for 1 h with Giemsa stain (5.0 ml of stock stain + 4.0 ml methanol + 91 ml phosphate buffer, pH 6.8), washed and mounted in DPX. One hundred good metaphase spreads were examined per animal and chromosome aberrations were classified into the general categories of breaks/gaps, exchanges and sister chromatid unions. Gaps have not been considered for statistical analysis of the data due to their controversial genetic significance[62].

Micronucleus assay

The micronuclei were analyzed in bone marrow and peripheral blood cells following the method of Schmid[63] and earlier used by us [31, 56]. Briefly, after 30 h of CP treatment, both the femora of mice were removed and bone marrow was flushed into a centrifuge tube with 1% sodium citrate solution (20^0C). Bone marrow cells were dispersed by gentle pipetting and immediately centrifuged (1000xg, 5

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min at 4°C). The cell pellet was resuspended in a small volume of 5% fetal calf serum in PBS. A drop of the suspension was smeared on a clean slide, air-dried and fixed in absolute methanol. The peripheral blood samples were also collected by snipping off the end of the tail of mice under the same treatment conditions and immediately blood smears were prepared. The smears were then air-dried, fixed in absolute methanol for 15 min. On the following day, fixed slides were stained with May-Grunwald and Giemsa combination. Two thousand each of polychromatic erythrocytes (PECs, immature erythrocytes, pink to purple in colour), normochromatic erythrocytes (NCEs, mature erythrocytes, yellowish-orange in colour), reticulocytes (RETs, purple-blue) and immature white cells (reddish-blue) were analyzed for the presence of micronuclei. Only rounded bodies approximately one-fifth to one-twentieth in size lying closer and possessing a similar staining intensity to that of the main nucleus, were scored as micronuclei.

RESULTS

Host survival patterns

Following tumor transplantation, an early sign of tumor development was noted from the 3rd day onwards. Control tumor-transplanted mice survived for 19-21 days. In the group of mice treated with AA or CP alone, mean survival time was increased to about 39 days and 54 days respectively. The hosts survivability was further enhanced to more than 68 days in the group of mice treated with AA plus CP (Table 1; Figure 1).

Table 1.
Antitumor activity of cyclophosphamide and ascorbic acid against murine ascites Dalton's lymphoma used singly or in combination.

Treatment	Day of treatment	Route of treatment	SURVIVAL DAYS (MEAN ± SD)	ILS (%)
Group-I (Control)	-	-	19.00 ± 2.5	-
Group-II (AA)	5th day	Oral	39.25 ± 3.20[a]	106.58
Group-III (CP)	10th day	i.p	54.80 ± 2.8[a,b]	188.42
Group-IV (AA + CP)	5th day 10th day	Oral i.p	68.00 ± 1.13[a,b,c]	257.89

Values represent the mean ± SD ; Control = Untreated tumourous, mice; AA = Ascorbic acid, CP= cyclophosphamide; treatments schedule and calculation of I.L.S. (%) as described in Materials and methods.
Students's *t*-test, n = 5; [a]P ≤ 0.001as compared to control, [b]P ≤ 0.001as compared to group-II, [c]P ≤ 0.001as compared to group-III.

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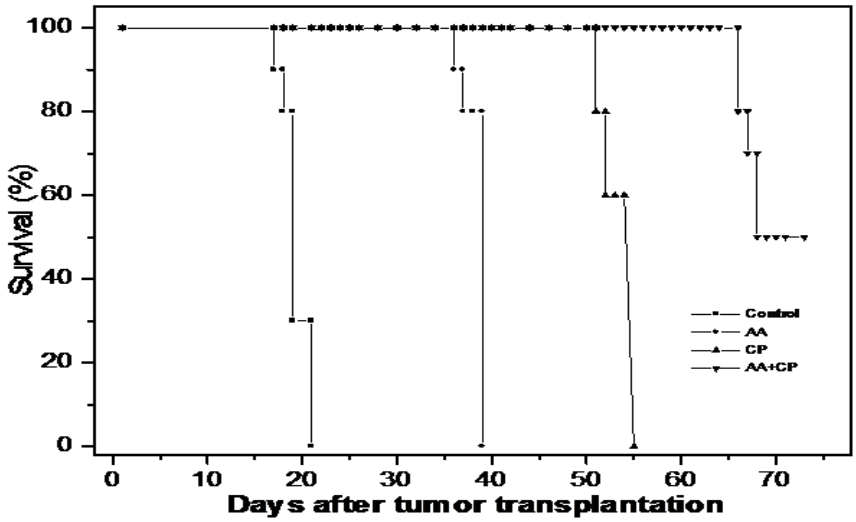

Figure 1. Mean survival pattern of Dalton's lymphoma-bearing mice under different treatment conditions. Group-I. Control = mice kept on normal tap water and received normal saline only. Group-II. 1% ascorbic acid (AA) was given through drinking water from 5th day post tumor transplantation, Group-III. cyclophosphamide (i.p.) at a dose of 200 mg/Kg body weight was administered on the 10th day post tumor transplantation; Group-IV. 1% ascorbic acid (AA) was given through drinking water from 5th day post tumor transplantation, and cyclophosphamide (i.p.) was administered on the 10th day post tumor transplantation.

Tumor pH

As compared to the control group, the tumor pH was significantly decreased at 72-96 hr after CP or AA plus CP treatment of mice (Table 2).

Table 2.

Average tumor pH in the ascites tumor from the tumor- bearing mice after treatment with ascorbic acid (AA) alone or in combination with cyclophosphamide (CP)

Treatment condition	Tumor pH
Group-I (Control)	6.87 ± 0.38
Group-II (AA treated)	6.34 ± 0.27
Group-III	
CP treated (24 hrs)	6.90 ± 0.41
CP treated (48 hrs)	6.80 ± 0.22
CP treated (72 hrs)	6.31 ± 0.18*
CP treated (96 hrs)	6.22 ± 0.30*
Group-IV	
AA+CP treated (24 hrs)	6.93 ± 0.26

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AA+CP treated (48 hrs)	6.78 ± 0.34
AA+CP treated (72 hrs)	6.30 ± 0.29*
AA+CP treated (96 hrs)	6.25 ± 0.20*

Values represent the mean ± SD. Students's *t*-test, n = 5; *P ≤ 0.01as compared to control, CP = cyclophosphamide; AA = ascorbic acid. The details about the treatment schedule has been described in Materials and Methods.

GSH level

GSH level of the Dalton's Lymphoma cells increased significantly during 4 to 15 days of tumor growth *in vivo* (Figure 2). CP alone or AA plus CP treatment of mice resulted in a decrease of TGSH level in DL cells (Table 3; Figure 2).

Table 3

Changes in protein, glutathione, lactate dehydrogenage activity and lipid peroxidation in Dalton's lymphoma tumor cells from the mice treated with cyclophosphamide alone or in combination with ascorbic acid

Treatment Condition	Protein		Glutathione (µmol/g wet wt.)	LDH activity (Units/mg protein)		LPO (nmol/mg protein)
	DL (mg/g wet wt.)	SN (mg/ml)		DL	SN	
Group-I (TB, Control)	153.84±9.05	39.86±2.51	4.63±0.16	0.38±0.017	0.23±0.02	0.12±0.008
Group-II (AA)	128.5±2.52*	30.95±1.02	3.07±0.25	0.45±0.03	0.25±0.023	0.17±0.012
Group-III						
CP 24h	132.4±2.45*	32.2±2.13	2.25±0.13*	0.32±0.02	0.39±0.015*	0.23±0.014*
CP 48h	120.5±4.41*	28.4±1.27	2.75±0.11*	0.26±0.023*	0.46±0.03*	0.28±0.016*
CP 72h	112.56±2.52*	22.6±2.3*	2.97±0.17*	0.21±0.03*	0.53±0.035*	0.32±0.024*
CP 96h	103.8±3.09*	18.2±1.78*	3.82±0.26	0.24±0.025*	0.50±0.027*	0.40±0.021*
Group-IV						
AA+ CP 24h	115.75±3.11*	28.2±0.68	2.38±0.13*	0.39±0.026	0.33±0.025*	0.22±0.015*
AA+ CP 48h	97.19±4.97*, a	24.6±1.25*	2.77±0.12*	0.33±0.03*	0.43±0.028*	0.27±0.023*

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AA+ CP 72h	85.8±4.09*, [a]	20.3±1.71*	3.18±0.18	0.27±0.02*	0.35±0.02*,[a]	0.25±0.024*
AA+ CP 96h	80.3±3.80*, [a]	17.5±1.50*	3.98±0.36	0.30±0.03*	0.26±0.025[a]	0.21±0.031*, [a]

The results are expressed as Mean ± S.D. Student's t- test, n=6, as compared to the respective control, *p ≤ 0.02; [a]P ≤ 0.01, as compared to group-III, at corresponding time of treatment. TB = Tumor-bearing mice; CP = cyclophosphamide; AA = ascorbic acid; DL=Dalton's Lymphoma tumor cells; SN= DL ascites supernatant; t-GSH = total glutathione; LDH= Lactate dehydrogenase; LPO= Lipid peroxidation; AA = ascorbic acid; The details about the treatment schedule has been described in Materials and Methods.

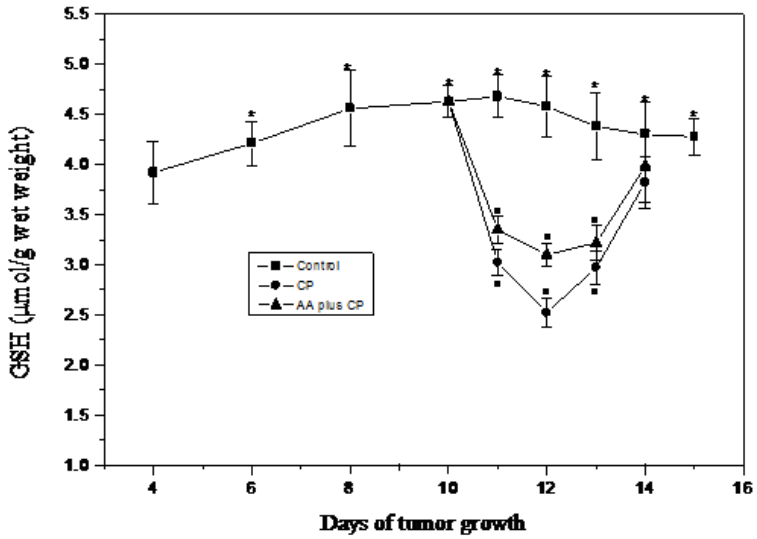

Figure 2. Changes in total GSH (TGSH) level in DL cells at different stages of tumor growth *in vivo* and after different treatment conditions. The details of the treatment schedule has been described in Materials and Methods. Results are reported as mean ± SD. Students's t-test, n= 4-5. *P ≤ 0.05 compared to the day 4 of tumor growth.

Lipid peroxidation (LPO)

The LPO in DL cells was noted to be significantly increased after AA or AA plus CP treatment of the mice. However, as compared to that of the CP treatment alone (group-III), the combination treatment (group-IV) showed a decrease of LPO at 96 h only (Table 3).

LDH activity

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The treatment of mice with cyclophosphamide alone (group-III) or combination treatment (group-IV) decreased the LDH activity in tumor cells with a concomitant increase of LDH in ascites supernatant (Table 3).

Chromosomal aberrations

Analysis of chromosomal aberrations (CA) in the bone marrow cells of tumor bearing mice revealed that CA increased after CP treatment as compared to controls. Both chromatid and isochromatid type gaps, chromatid deletions that include breaks, sister chromatid unions and exchanges were observed in the treated series of which chromatid breaks and gaps occurred more frequently. The total number of aberrations as well as the percent abnormal metaphases decreased appreciably with the time of treatment from 24 h to 96 h. A comparative analysis at corresponding periods of treatment revealed that total abnormal metaphases decreased significantly in ascorbic acid plus CP treated mice (group-IV) as compared to those receiving CP alone (group-III) (Table 4).

Table 4.

Frequency of chromosomal aberrations in bone marrow cells of mice under different treatment conditions

Treatment	No of metaphases scored	Mean % of aberrant metaphases	Chromatid %		Exchange %	Isochromatid %		SCU%	Total aberrations per cell Mean ± SD
			Breaks	Gaps		Breaks	Gaps		
TB Control	500	0.76	0.25	0.31	0.28	-	-	-	0.530.08
AA	500	0.83	0.28	0.46	0.30	-	-	-	0.58±0.11
CP24h	400	44.25	32.15	14.05	6.4	4.0	4.32	2.10	44.65±5.3 *#
CP48h	400	35.0	20.6	8.3	4.1	2.3	2.52	1.60	28.6±3.88 *#
CP72h	400	23.5	12.3	4.2	2.7	1.2	2.32	1.52	17.72±2.70 *#
CP96h	300	14.0	4.8	1.8	2.1	0.8	1.09	0.34	8.04±0.97 *#
AA+CP24h	300	25.5	27.9	8.6	6.12	2.56	3.85	1.25	37.83±4.42 *#
AA+CP48h	300	11.5	14.3	4.2	3.08	1.93	2.56	1.83	21.14±2.14 *#
AA+CP72h	300	7.03	3.74	2.0	1.83	0.94	1.86	1.24	7.75±1.03 *#
AA+CP96h	300	2.74	1.38	0.8	0.93	0.41	0.43	0.56	3.28±0.068 *#

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TB = Tumor-bearing mice; CP=Cyclophosphamide was given as a single dose (200mg/kg body wt.i.p.).AA= Ascorbic acid (1%) was given in the drinking water on the5th day after tumor transplantation, SCU, Sister chromatid unions. Gaps have not been included .Results are expressed as Mean± SD. Student',s t-test, n=4-5; as compared to the control(*) and to respective treatment with ascorbic acid alone(#) , *# $p \leq 0.05$.

Micronucleus assay

The frequency of micronucleated cells in the bone marrow and peripheral blood of mice treated with cyclophosphamide (CP) increased significantly (Table 5; Figure 3). However, as compared to that of the group treated with CP alone (group-III), in the group of mice treated with ascorbic acid and CP combination (group-IV), the frequency of micronucleated cells decreased significantly (Table 5; Fig. 3).

Table 5.

Percentage of micronuclei in bone marrow and peripheral blood of mice under different treatment conditions in vivo.

Treatment	Bone marrow			Peripheral blood		
	PCEs	NCEs cells	Nucleated	RETs	NCEs cells	Nucleated
Control	0.13 ± 0.06	0.06 ± 0.03	0.16 ± 0.05	0.05 ± 0.02	0.02 ± 0.01	0.08 ± 0.02
CP	1.67 ± 0.21*	0.78 ± 0.15*	0.62 ± 0.08*	0.70 *± 0.19	0.21 ± 0.09*	0.30 ± 0.06*
Vitamin C	0.28 ± 0.05*	0.18 ± 0.04*	0.15 ± 0.06	0.11 ± 0.04	0.04 ± 0.01	0.05 ± 0.01
Vitamin C + CP	0.45 ± 0.10*, a	0.22 ± 0.08*, a	0.18 ± 0.08a	0.19 ± 0.08*, a	0.13 ± 0.04*	0.12 ± 0.03a

CP: Cyclophosphamide was administered i.p. at a single dose of 200mg/kg body weight, on the 10th day post tumor transplantation. Vitamin C (ascorbic acid, 1%) was given through drinking water from the 5th day to 10th day of tumor transplantation. PCEs = Polychromatic erythrocyte; NCEs = Normochromatic erythrocyte; RETs = Reticulocytes. Results are expressed as mean ± S.D. in percentage. For each treatment condition, 2000 cells were examined.

* Student's *t*-test, n = 5, as compared to respective control.

[a] Student's *t*-test, n = 5, as compared to respective treatment with CP alone. $*, {}^{a}P \leq 0.05$

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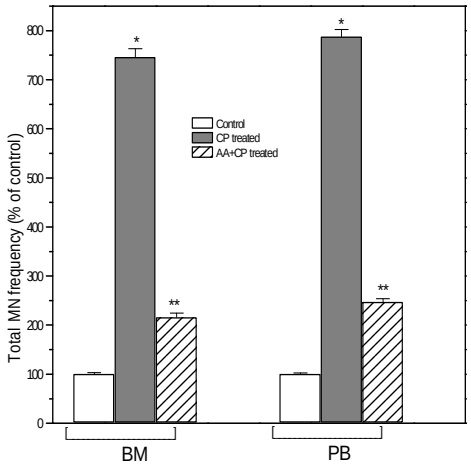

Figure 3. Changes in the frequency (%) of total micronuclei (MN) in the cells. BM = Bone marrow; PB= Peripheral blood. AA = ascorbic acid; DL=Dalton's Lymphoma tumor cells. The details about the treatment schedule has been described in Materials and Methods. *Student's *t*-test, as compared to respective control. **Student's *t*-test, as compared to respective treatment with CP alone. *, ** $P \leq 0.001$

DISCUSSIONS

Cyclophosphamide, an alkylating agent with immunosuppressive activities, is used in the treatment of malignant tumors[2,3,6] and for organ transplantation[9]. It is a prodrug which is converted by cytochrome P450 enzymes in the liver to active metabolites. The main active metabolite is 4-hydroxycyclophosphamide, which exists in equilibrium with its tautomer, aldophosphamide. Most of the aldophosphamide is oxidised by the enzyme aldehyde dehydrogenase (ALDH) to make carboxyphosphamide. A small proportion of aldophosphamide is converted into active DNA cross- linking metabolite phosphoramide mustard and acrolein which exert therapeutic effects[12, 13].

The results of the hosts survival data of antitumor activity experiments in present studies reveal that CP is quite effective against ascites Dalton's lymphoma in mice, showing the increase in life span (ILS) by about 188% as compared to control (Table 1). This corroborates the antitumor properties of CP. CP treatment has also been associated with the induction of oxidative stress[64] and the potential use of dietary antioxidants to reduce the activity of free-radical induced reactions has drawn increasing attention[65-67]. Here the dietary antioxidant in the form of vitamin C or ascorbic acid (AA) was used, and its combination with CP showed further increase in hosts survivality (ILS ~ 258%) which is significantly more than that in the group of mice treated with either agent alone (Table 1, Figure 1). It has been reported that the i.p. administration of vitamin C and vitamin K3 produced a distinct chemotherapy-potentiating effect for cyclophosphamide, especially when injected before chemotherapy, on the survival of ascites liver tumor-bearing mice and this potentiating treatment did not increase the general and organ toxicity[68]. In present studies also, the combined antitumor activity of AA and CP appears to be sequence dependent because when mice were treated with CP first on the 10th day

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and, then AA was given for 4 to 5 days, the hosts survival rates were almost similar to the group treated with CP alone (ILS 191%; unpublished results). Therefore, the treatment of tumor-bearing hosts with AA first could be helpful in developing suitable conditions in the host to potentiate therapeutic effect of CP and enhanced effective tumor regression. It has been reported that ascorbic acid supplementation of cells positively modulated Mut L homologue-1 (MLH1) and p73 genes and increased their susceptibility to apoptosis by DNA-damaging agent cisplatin[69]. The analysis of tumor pH (extracellular) showed slight acidic pH (pH 6.87), and after CP treatment alone or in combination treatment with AA (group IV), it significantly decreased, particularly at 72-96 h (Table 2). It has been known that the microenvironment in tumors is acidic as compared with that in normal tissues because of elevated anaerobic as well as aerobic glycolysis in tumors [70, 71]. The low tumor pH has been suggested to help the better response of tumors to various treatments such as chemotherapy, radiotherapy and hyperthermia. However, chemotherapeutic response against tumors depends on the nature of the drugs also. For instance, the acidic extracellular pH increases the cellular uptake of weakly acidic drugs such as cyclophosphamide and cisplatin, and thus, increases 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 [71]. Here also, the low tumor pH and its further decrease after CP or AA plus CP treatment may facilitate the chemotherapeutic response of tumor to CP chemotherapy. It has been established that hypoxic environment up regulates a number of transcription factors such as HIF-1[72, 73]. 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 [74, 75]. It has been observed that exposure of tumor cells to a low pH medium elevates significantly p53 and p21 expression[76].

CP treatment of mice either alone or in combination with AA caused a significant decrease in total protein level in the DL tumor cells (Table 3). This decrease in protein level may involve inhibited protein synthesis and or proteolysis by peptidases within the cells. This is in agreement with earlier reports indicating that cyclophosphamide[12] and other alkylating agents[77] have an inhibitory effect on protein synthesis. Decrease in protein content in the testes of cyclophosphamide treated albino rats has also been reported and suggested that this decrease may be due to cell damage[78]. Lactate dehydrogenase (LDH, EC 1.1.1.27), the terminal enzyme in anaerobic glycolysis, catalyzes a reversible reaction of pyruvate to lacate. Following CP or AA plus CP treatment of tumor bearing hosts, an overall decrease in LDH activity was noted in tumor cells with its corresponding increase in the ascites supernatant(Table 3). The decrease in LDH activity in tumor cells may indicate decreased synthesis and/or increased leakage of the enzyme from the cells due to cell damage/death. The second possibility may be more implicated here, since, simultaneously an increase of LDH level/activity was observed in the ascites supernatant also. In fact, leakage of LDH from the cells into the medium and blood serum has been suggested to indicate the cell injury/damage[79-81].

Glutathione, an endogenous intracellular thiol-containing tripeptide (L-γ-glutamyl-L-cysteinyl-glycine), is an important cellular antioxidant and has been the focus of interest in cancer chemotherapy[82,83]. Under normal physiological conditions, more than 98% of glutahione exists in

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reduced form (GSH). It is involved directly or indirectly in a variety of biological phenomena including the bioreductive reactions, maintenance of enzyme activity, amino acid transport, protections against oxidative stress, detoxification of xenobiotics, and drug metabolism[84]. Determination of GSH in DL cells, during early, mid and later periods of tumor growth showed that total GSH level in DL cells increased significantly with tumor growth with slight decrease later during 12-15 days when tumor growth was declining (Figure 2). It has been reported that GSH levels increase in Ehrlich ascites tumor cells also when they proliferate actively, reaching a maximum approximately on day 7 and decreasing later during the 14th day of tumor growth which was correlated with a decrease in cell proliferation and in the rate of GSH synthesis[85]. Cancer cells can generate large amounts of hydrogen peroxide which may contribute to their ability to mutate and damage normal tissues[86]. The observed increase in GSH level in the DL cells may suggest its involvement to facilitate proliferation and metabolism of tumor cells in the host and agrees with the report that elevation of intracellular GSH in tumor cells is associated with mitogenic stimulation[87] and that GSH controls the onset of tumor cell proliferation by regulating protein kinase C activity and intracellular pH[88]. After the treatment of tumor-bearing mice with CP, total GSH levels decreased very significantly and it could lead to alteration in the cellular antioxidant machinery, less protective mechanisms and increasing CP-induced cytotoxic effect on DL cells. In combination treatment of AA with CP, slight increase in GSH level was noted in Dl cells, but still it was quite low as compared to controls (Figure 2). It has been reported that the tissue ascorbate levels of GSH-deficient animals were greatly increased by giving ascorbate, as expected, but, giving ascorbate also led to higher GSH levels and suggested that ascorbate and GSH can spare each other under appropriate experimental conditions[89]. CP treatment caused an increase in LPO also in DL cells and supports the earlier observations on the CP-induced LPO in other experimental condition[90]. As compared to CP alone, the combination treatment did not cause the increase of LPO, instead showed a decrease of LPO at 96 hr of treatment, but it was still significantly more than that of control (Table 3). This increase in LPO in DL cells may also be facilitating the tumor cell lysis along with the decrease in GSH level in Dl cells after CP or AA plus CP treatment.

Cyclophosphamide is a potent antitumor drug. However, as with most such agents, with CP also, there can be the development of different side effects [36, 91] with the possible induction of mutagenic effects in non-tumor cells in the hosts [21, 30, 92]. The use of dietary antioxidants to prevent antitumor agent-induced chromosomal damage in non-tumor cells has elicited much interest[32]. In present studies, development of chromosomal aberrations as well as micronuclei in bone marrow cells and peripheral blood cells were observed after CP treatment of tumor-bearing mice (Table 4 and 5) and it is in agreement with the earlier reports showing the mutagenic/genotoxic properties of CP [21-23, 30, 92]. The analysis of the pattern of the chromosomal aberrations revealed a higher frequency of chromatid breaks and gaps. The chromosomal aberrations were noticed to be highest at 24 h of CP treatment which decreased gradually during later periods. This decrease in the frequency of chromosomal aberrations during later periods (72-96 h) of CP treatment may be due to a post-replication repair process. The involvement of post-replication repair has been suggested to be involved in other cases also[93]. However, as compared to CP treated group, in the group of mice treated with AA and CP combination, chromosomal aberrations were significantly less (Table 4). The changes in other

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mutagenic parameter i.e. development of micronuclei in bone marrow cells as well as peripheral blood cells also shows similar pattern (Table 5; Figure 3)) as observed for chromosomal aberrations. Micronuclei are cytoplasmic chromatin masses with the appearance of small nuclei that arise from chromosome fragments of intact whole chromosomes lagging behind at the anaphase stage cell division[21] and they can be easily recognized in the cytoplasm of immature polychromatic erythrocytes [94]. In present studies, the effect of ascorbic acid on CP-induced micronuclei frequency was assessed by the analysis of micronuclei in polychromatic erythrocytes (PCEs), normochromatic erythrocytes (NCEs) and the nucleated cells in the bone marrow cells of the hosts. In comparison to the untreated control, the micronuclei increased significantly in PCEs and NCEs in the bone marrow cells as well as in RETs and NCEs in the peripheral blood after cyclophosphamide treatment, thereby, indicating its mutagenic potentials in the hosts (Table 5; Figure 3) and supporting other findings on the ability of CP to induce micronuclei in cells[95]. However, the frequency of micronuclei was observed to decrease significantly in the group of mice supplemented with ascorbic acid as compared to CP treatment alone (Fig. 3). The antioxidant properties of ascorbic acid could be an important factor in the protection against CP-induced DNA damages and tissue toxicity[96] and ascorbic acid has been reported as an anti-sister chromatid exchanges induced by cyclophosphamide in mice[30]. Earlier also the protective role of vitamin C against the mutagenic effects of various chemical agents i.e. cisplatin[31], norethynodrel[33] mitomycin C[92], N-ethyl-N'-nitrosourea[97], bleomycin[30] as well as cyclophosphamide[30] has been reported. In case of the important cellular antioxidant, reduced glutathione (GSH), it has been suggested that increasing cellular GSH level may prevent the formation of active metabolites of CP, i.e phosphoramide mustard, thus, modifying/lowering CP-induced micronuclei in cells[21].

CONCLUSION

The results of the present investigation depict the protective effect of ascorbic acid against the mutagenic effects by cyclophosphamide in Dalton's lymphoma tumor-bearing mice. In context with present findings, this could be very significant as the combination treatment of AA plus CP has also been noted to be useful in enhancing CP-mediated therapeutic efficacy. Thus, it is suggested that as compared to cyclophosphamide alone, ascorbic acid plus cyclophosphamide combination chemotherapy could be very helpful in enhancing cyclophosphamide-mediated therapeutic efficacy and at the same time decreasing its mutagenic potentials in the tumor-bearing hosts.

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REFERENCES

1. Bergsagel DE, Robertson GL and Hasselback R, Effect of cyclophosphamide on advanced lung cancer and the hematological toxicity of large, intermittent intravenous doses, Can Med Assoc J, 98: 532–538, (1968).

MODULATORY EFFECT OF ASCORBIC ACID (VITAMIN C) ON CYCLOPHOSPHAMIDE-MEDIATED ANTITUMOR ACTIVITY AND MUTAGENICITY IN MICE BEARING ASCITES DALTON'S LYMPHOMA

2. Ahmed AR and Hombal SM, Cycophosphamide (Cytoxan). A review on relevant pharmacology and clinical uses, J Am Acad Dematol, 11: 1115-1126, (1984).
3. Moore MJ, Clinical pharmacokinetics of cyclophosphamide, Clin Pharmacokinet, 20: 194 – 208, (1991).
4. Telegin L Yu, Pisarev VM and Pevnitsky LA, Cyclophosphamide enhances the immunosuppressive action of its own active metabolites, Doklady Biological Sci, 423: 437-439, (2008).
5. Black DJ and Livingston RB, Antineoplastic drugs in 1990-a review (part I), Drugs, 39:489-501, (1990).
6. Khan TS, Sundin A, Juhlin C, Wilander E, Oberg, K and Eriksson B, Vincristine, cisplatin, teniposide, and cyclophosphamide combination in the treatment of recurrent or metastatic adrenocortical cancer,Med Oncol, 21:167-178, (2004).
7. Perini P, Calabrese M, Rinaldi L and Gallo P, Cyclophosphamide-based combination therapies for autoimmunity, Neurological Sci, 29:233-234, (2008).
8. Suwa A, Hirakata M, Satoh S, Mimori T, Utsumik K and Inada S, Rheumatoid arthritis associated with methotrexate-induced pneumonitis : Improvement with cyclophosphamide therapy, Clinical Exp Rheumatol, 17: 355-358, (1999).
9. Starzl TE, , Penn I, Schroter G, Putnam CW, Halgrimson CG, Martineau G, Amemiya H and Groth CG, Cyclophosphamide and human organ transplantation. The Lancet, 298: 70 - 74, (1971).
10. Eto M, Nishimura Y, Matsuo K, Omoto K, Goto K, Kumuzawa J and Nomoto K, Prolongation of kidney graft survival by cyclophsophamide-induced tolerance in rats, J Urol, 153 : 1693-1696, (1995).
11. Kang HJ, Shin HY, Choi HS and Ahn HS, Fludarabine, cyclophosphamide plus thymoglobulin conditioning regimen for unrelated bone marrow transplantation in severe aplastic anemia. Bone Marrow Transplant, 34: 939-943, (2004).
12. Fleming RA, An overview of cyclophosphamide and ifosfamide pharmacology, Pharmacother, 17:146S-154S, (1997).
13. Huang Z, Raychowdhury MK and Waxman DJ, Impact of liver P450 reductase suppression on cyclophosphamide activation, pharmacokinetics and antitumoral activity in a cyctochrome P450-based cancer gene therapy model, Cancer gene Therapy, 7:1034-1042, (2000).
14. Baumann F and Preiss R, Cyclophosphamide and related anticancer drugs, J Chromatogr B: Biomed Sci Appl, 764:173-192, (2001).
15. Cyclophosphamide side effects. Available at http://www.drugs.com/sfx/cyclophosphamide-side-effects.html (Accessed September 15, (2009)
16. Fraiser LH, Kanekal S and Kehrer JP, Cyclophosphamide toxicity. characterising and avoiding the problem, Drug, 42: 781-795, (1991).
17. Ayhanci A, Uyar R, Aral E, Kabadere S and Appak S, Protective effect of zinc on cyclophosphamide-induced hematoxicity and urotoxicity, Biol. Trace Element Res, 126:186-193, (2008).
18. Travis LB, Curtis RE, Glimelius B, Holowaty EJ, Van Leeuwen FE, Lynch CF, Hagenbeek A, Stovall M, Banks PM, Adami J, Gospodarowicz MK, Wacholder S, Inskip PD, Tucker MA and Boice JDJr, Bladder and kidney cancer following cyclophosphamide therapy for non-Hodgkin's Lymphoma, J Natl Cancer Inst, 87:524-531, (1995).

MODULATORY EFFECT OF ASCORBIC ACID (VITAMIN C) ON CYCLOPHOSPHAMIDE-MEDIATED ANTITUMOR ACTIVITY AND MUTAGENICITY IN MICE BEARING ASCITES DALTON'S LYMPHOMA

19. Talar-Williams C, Hijazi Y, Walther MM, Linehan WM, Hallahan CV, Lubensky I, Kerr GS, Hoffman GS, Fauci AS and Sneller MC, Cyclophosphamide-induced cystitis and bladder cancer in patients with Wegener granulomatosis, Ann Intern Med, 124: 477-484, (1996).
20. Nilsson S and Ullén A, Chemotherapy-induced bladder cancer, Scand J Urol Nephrol, 42(Suppl 218): 89-92, (2008).
21. Czyzewska A and Mazur L, Suppressing effect of WR-2721 on micronuclei induced by cyclophosphamide in mice, Terato Carcino Mutagen, 15: 109-114, (1995).
22. Takeshita T and Conner MK, Persistence of cyclophosphamide-induced damage in bone marrow as indicated by sister chromatid exchange analysis, Carcinogenesis, 6: 1097-1102, (1985).
23. Novotná B and Jelínek R, Mutagenic and teratogenic effects of cyclophosphamide on the chick embryo: Chromosomal aberrations and cell proliferation in affected and unaffected tissues, Teratogenesis Carcinog Mutagen, 10: 341-350, (2003).
24. DF Holloway and FJ Peterson. Ascorbic acid in drug metabolism, In: D.A. Roe and T.C. Campbell (eds.), *Drugs and Nutrients: The interactive effects*, Marcel Dekker, New York and Basel, 1984, pp. 225-295.
25. Henson DE, Block G and Levine M, Ascorbic acid: biologic functions and relation to cancer, J Natl Cancer Inst, 83: 547-550, (1991).
26. Iqbal K, Khan A and Khattak MMAK, Biological significance of ascorbic acid (Vitamin C) in human health – a review, Pakistan J Nutr, 3: 5-13, (2004).
27. Walingo KM, Role of vitamin C(ascorbic acid) on human health- a review, African J Food Agri Nutritional Development, 5: 1-12, (2005).
28. Ghosh J and Das S, Evaluation of vitamin A and C status in normal and malignant conditions and their possible role in cancer prevention, Jpn J Cancer Res,76:1174 – 1178, (1985).
29. Prasad SB, Giri A and Arjun J, Use of subtherapeutical dose of cisplatin and vitamin C against murine Dalton's lymphoma, Pol J Pharmacol Pharm, 44: 383-391, (1992).
30. Vijayalaxmi KK and Venu R, In vivo anticlastogenic effects of L-ascorbic acid in mice, Mutat Res, 438: 47-51, (1999).
31. Giri A, Khynriam D and Prasad SB, Vitamin C mediated protection on cisplatin induced mutagenicity in mice. Mutat Res, 421: 139-148, (1998).
32. Antunes LMG, Araujo MCP, Darin J.D'Arc C and Bianchi M.deL.P, Effects of antioxidants curcumin and vitamin C on cisplatin-induced clasogenesis in Wistar rat bone marrow cells. Mutat Res, 465: 131-137, (2000).
33. Siddique YH, Beg T and Afzal M, Anticlastogenic effects of ascorbic acid against the genotoxic damage induced by norethynodrel, Advances Env Biol, 1(1): 27-32, (2007).
34. Kola I, Vogel R and Spielman H, Co-administration of ascorbic acid with cyclophosphamide (CPA) to pregnant mice inhibits the clastogenic activity of CPA in preimplantation murine blastocysts, Mutagenesis, 4: 297-301, (1989).
35. Antunes LMG, Darin JD and Bianchi MD, Protective effects of vitamin C against cisplatin-induced nephrotoxicity and lipid peroxidation in adult rats: a dose-dependent study, Pharmacol Res, 41: 405-411, (2000).
36. Ghosh S, Ghosh D, Chattopadhyay S and Debnath J, Effect of ascorbic acid supplementation on liver and kidney toxicity in cyclophosphamide-treated female albino rats, J Toxicol Sci, 24: 141-144, (1999).

MODULATORY EFFECT OF ASCORBIC ACID (VITAMIN C) ON CYCLOPHOSPHAMIDE-MEDIATED ANTITUMOR ACTIVITY AND MUTAGENICITY IN MICE BEARING ASCITES DALTON'S LYMPHOMA

37. Chang SI, Bohwan J, Pilju Y, Changbo P, Jung-Duck P and Doug-Young R, Arsenic-induced toxicity and the protective role of ascorbic acid in mouse testis, Toxico Appl pharmacol, 218: 196-203, (2007).
38. Olas B, Wachowicz B and Buczynski A, Vitamin C suppresses the cisplatin toxicity on blood Platelets, Anticancer Drugs, 11: 487-493, (2000).
39. Yasmina M and Abdennour C, Influence of vitamin C on testicular functions of domestic rabbit *Oryctolagus Cuniculus* under mercury exposure, European J Sci Res, 22: 197-204, (2008).
40. Kathleen A, Ascorbic acid in the prevention and treatment of cancer, Altern Med Rev, 3:174-186, (1998).
41. Verrax J and Buc Calderon P, The controversial place of vitamin C in cancer treatment, Biochem Pharmacol, 1644-1652, (2008).
42. Cameron E and Pauling L, Supplemental ascorbate in the supportive treatment of cancer: Reevaluation of prolongation of survival times in terminal human cancer, Proc Natl Acad Sci, 75: 4538-4542, (1978).
43. Murata A, Morishige F and Yamaguchi H, Prolongation of survival times of terminal cancer patients by administration of large doses of ascorbate. Int J Vitam Nutr Res Suppl, 23: 103-113, (1982).
44. Ohno S, Ohno V, Suzuki N, Soma, GI and Inoue M, High-dose vitamin C (Ascorbic Acid) therapy in the treatment of patients with advanced cancer, Anticancer Res, 29(3): 809- 815, (2009).
45. Leibovitz B, Schlesser J: Effect of L-ascorbic acid on leukemia development and breast cancer in various inbred strains of mice. In: F.L. Meyskens and K.N. Prasad (eds.), *Modulation and mediation of cancer by vitamins*, Karger, Basel, 1983, pp140-143.
46. Creagan ET, Moertel CG, O'Fallon JR, Schutt AJ, O'Connell MJ, Rubin J and Frytak S, Failure Of high-dose vitamin C (ascorbic acid) therapy to benefit patients with advanced cancer. a controlled trial, New Engl J Med, 301: 687–690, (1979).
47. Slaga TJ and Bracken WM, The effects of antioxidant on skin tumor initiation and aryl hydrocarbon hydroxylase. Cancer Res, 37:1631-1635, (1977).
48. Dunham WB, Zuckerkandl E, Reynolds R, Willoughby R, Marcuson R, Barth R and Pauling L, Effects of intake of L-ascorbic acid on the incidence of dermal neoplasms induced in mice by ultraviolet light, Proc Natl Acad Sci (USA),79: 7532-7536, (1982).
49. Lee KW, Lee HJ, Kang KS and Lee CY, Preventive effects of vitamin C on carcinogenesis, Lancet, 359: 172, (2002).
50. Surjyo, B and Anisur Rahman KB, Protective action of an anti-oxidant (L-Ascorbic acid) against genotoxicity and cytotoxicity in mice during p-DAB-induced hepatocarcinogenesis, Indian J Cancer, 41: 72-80, (2004).
51. Banic S, Vitamin C acts as a co-carcinogen to methylcholanthrene in guinea pigs, Cancer Lett, 11: 239-242, (1981).
52. Fukushima S, Imaida K, Sakata T, Okamura T, Shibata M and Ito N, Promoting effects of Sodium L-ascorbate on two stage urinary bladder carcinogenesis in rats. Cancer Res, 43:

MODULATORY EFFECT OF ASCORBIC ACID (VITAMIN C) ON CYCLOPHOSPHAMIDE-MEDIATED ANTITUMOR ACTIVITY AND MUTAGENICITY IN MICE BEARING ASCITES DALTON'S LYMPHOMA

4454 - 4457, (1983).
53. Frith CH, Rule J and Kodell RL, The effects of ascorbic acid on the induction of urothelial lesions in mice by 2-acetylaminofluorene, Toxicology, 6: 309-318, (1980).
54. Shamberger RJ, Genetic toxicology of ascorbic acid, Mutat Res, 133-135, (1984).
55. Nefic H, The genotoxicity of vitamin C in vitro, Bosnian J Basic Med Sci, 8(2): 141-146, (2008).
56. Giri S, Giri A, Sharma GD and Prasad SB, Mutagenic effects of carbosulfan, a carbamate pesticide, Mutat Res, 519: 75-82, (2002).
57. Nicol BM and Prasad SB, Sialic acid changes in Dalton's lymphoma-bearing mice after cyclophosphamide and cisplatin treatment, Braz J Med Biol Res, 35: 549-553, (2002).
58. Sedlak J and Lindsay RH, Estimation of total, protein-bound and non-protein sulfhydryl groups In tissue with Ellman's reagent, Anal Biochem, 25: 192-205, (1968).
59. Buege JA and Aust SD, Mirosomal lipid proxidation. Methods Enzymol, 52: 302-310, (1978).
60. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent, J Biol Chem, 193: 265-275, (1951).
61. Cabaud, P J and F Wro´blewski, Colorimetric measurement of lactic dehydrogenase activity of body fluids, Am J Clin Pathol, 30: 234–236, (1958).
62. Preston RJ, Au W, Bender MA, Brewen JG, Carrano AV, Heddle JA, McFee AF, Wolff S and Wassom JS, Mammalian in vivo and in vitro cytogenetic assays: a report of the USEPA's gene-tox programme, Mutat Res, 87: 143-188, (1981).
62. Vorhaben JE and Campbell JW, Glutamine synthetase: a mitochondrial enzyme in uricotelic Species, J Biol Chem, 247(9): 2763-2767, (1972).
63. Schmid W, The micronucleus test, Mutation Res, 31: 9 – 15, (1975).
64. Abraham P, Kanakasabapathy I and Preethi kulothungan P, Alterations in antioxidant enzyme activities and increased oxidative stress in cyclophosphamide- induced hemorrhagic cystitis in the rat, Cancer Therapy, 6: 563-570, (2008).
65. Abd El-Rahim AH and Hafiz NA, Investigation on the protective effect of grape seed and Linseed oils against cyclophosphamide induced genotoxicity in mice. Global Veterinaria 3 (5): 377-382, (2009).
66. McCall MR and Frei B, Can antioxidant vitamins materially reduce oxidative damage in humans? Free Rad Biol Med, 26: 1034-1053, (1999).
67. Lamson DW and Brignall MS, Antioxidants in cancer therapy; their actions and interactions with oncologic therapies, Altern Med Rev, 4(5): 304-329, (1999).
68. Taper HS, deGerlache J, Lans M and Roberfroid M, Non-toxic potentiation of cancer Chemotherapy by combined C and K3 vitamin pretreatment, Int J Cancer, 40: 575-579, (1987).
69. Catani MV, Costanzo A, Savini I, Levrero M, de Laurenzi V, Wang JY, Melino G and Avigliano L, Ascorbate up-regulates MLH1 (Mut L homologue-1) and p73: implications for the cellular response to DNA damage, Biochem J, 364: 441-447, (2002).
70. Song CW, Park HJ and Ross BD, Intra- and extracellular pH in solid tumors. In: B.V. Teicher, (ed), *Antiangiogenic adnets in cancer therapy*, Humana Press Inc, Totowa, 1998, pp 51–64.
71. Song CW, Griffin R and Park HJ, Influence of Tumor pH on therapeutic response. In :

MODULATORY EFFECT OF ASCORBIC ACID (VITAMIN C) ON CYCLOPHOSPHAMIDE-MEDIATED ANTITUMOR ACTIVITY AND MUTAGENICITY IN MICE BEARING ASCITES DALTON'S LYMPHOMA

B. Teicher (eds), *Cancer drug discovery and development: Cancer drug resistance*, Humana Press Inc., Totowa, 2006, pp 21-42.

72. Subarsky P and Hill RP, The hypoxic tumour microencironment and metastatic progression, Clin Exp Metastasis, 20: 237–250, (2003).
73. Le QT, Denko NC and Giaccia AJ, Hypoxic gene expression and metastasis, Cancer Metastasis Rev, 23: 293–310, (2004).
74. Semenza GL, Targeting HIF-1 for cancer therapy, Nat Rev Cancer, 3 :721–732, (2003).
75. Hong SS, Lee H and Kim KW: HIF-1a: a valid therapeutic target for tumor therapy, Cancer Res Treat, 36: 344–353, (2004).
76. Ohtsubo T, Wang X, Takahashi A, Ohnishi K, Saito H, Song CW and Takeo Ohnishi T, p53-dependent induction of WAF1 by low pH culture condition in human glioblastoma cells, Cancer Res, 57: 3910–3913, (1997).
77. Harder HC and Rosenberg B, Inhibitory effects of antitumor platinum compounds on DNA, RNA and protein synthesis in mammalian cells in vitro, Int J Cancer, 6: 207-216, (1970).
78. Kaur R, Sangha GK and Bilaspuri GS, Cyclophosphamide-induced structural and biochemical changes in testes and epididymidis of rats, Indian J Exp Biol, 35: 771-775, (1997).
79. Asztalos B and Nemcsok J, Effect of pesticides on the LDH activity and isozyme patterns of Carp (Cyprinus carpio L.) sera, Comp Biochem Physiol, 82C: 217-219, (1985).
80. Kim YK, Jung JS, Lee SH and Kim YW, Effects of antioxidants and Ca^{++} in cisplatin-induced cell injury in rabbit renal cortical slices, Toxicol Appl Pharmacol, 146: 261-269, (1997).
81. Prasad SB and Giri A, Effect of cisplatin on the lactate dehydrogenase activity and its isozyme pattern in Dalton's lymphoma bearing mice, Cytologia, 64: 259-267, (1999).
82. Arrick BA and Nathan CF, Glutathione metabolism as a determinant of therapeutic efficacy : A Review, Cancer Res, 44: 4224 - 4232, (1984).
83. Khynriam D and Prasad SB, Changes in endogenous tissue glutathione level in relation to Murine ascites tumor growth and the anticancer activity of cisplatin, Brazilian J. Med. Biol. Res, 36: 53-63, (2003).
84. Wang W and Ballatori N, Endogenous glutathione conjugates: occurrence and biological functions, Pharmacol Rev, 50: 335 – 355, (1998).
85. Estrela JM., Hernandez R, Terradez P, Asensi M, Puertes IR and Vina J, Regulation of Glutathione metabolism in Ehrlich ascites tumour cells, Biochem J, 286: 257-262, (1992).
86. Szatrowski TP and Nathan CF, Production of large amounts of hydrogen peroxide by human Tumor cells, Cancer Res, 51: 794-798, (1991).
87. Shaw JP and Chou IN, Elevation of intracellular glutathione content associated with mitogenic stimulation of quiescent fibroblasts, J Cell Physiol, 129: 193-198, (1986).
88. Terradez P, Asensi M, Lasso De La Vega MC, Puertes IR, Vina J and Estrela JM, Depletion of tumor glutathione in vivo by buthionine sulfoximine: modulation by the rate of cellular proliferation and inhibition of cancer growth, Biochemical J, 292 : 477 – 483, (1993).
89. Meister A, Glutathione-ascorbic acid antioxidant system in animals, J Biol Chem, 269: 9397-9400, (1994).

MODULATORY EFFECT OF ASCORBIC ACID (VITAMIN C) ON CYCLOPHOSPHAMIDE-MEDIATED ANTITUMOR ACTIVITY AND MUTAGENICITY IN MICE BEARING ASCITES DALTON'S LYMPHOMA

90. Chakraborty P, Ugir Hossain Sk, Murmu N, Das JK, Pal S and Bhattacharya S, Modulation of cyclophosphamide-induced cellular toxicity by diphenylmethyl selenocyanate *in vivo*, an enzymatic study, J Cancer Molecules, 4(6): 183-189, (2009).
91. Das UB, Mallick M, Debnath JM and Ghosh D, Protective effect of ascorbic acid on cyclophosphamide-induced testicular, gametogenic and androgenic disorders in male rats, Asian J Androl, 4: 201-207, (2002).
92. Krishna G, Nath J and Ong T, Inhibition of cyclophopsphamide and mitomycin C-induced sister Chromatid exchanges in mice by vitamin C, Cancer Res, 46: 2670-2674, (1986).
93. Sorenson CM and Eastman A, Mechanism of *cis*-diamminedichloroplatinum (II)-induced cytotoxicity: rule of G2 arrest and double strand brakes, Cancer Res, 48: 6703-6707, (1988).
94. Schmid W, The micronucleus test for cytogenetic analysis. In : Hollaender A. (Ed.), vol. 4, *Chemical mutagens: principles and methods for their detection*, Plenum Press, New York, 1976, pp31-53.
95. Hamada S, Yamasaki K, Nakanishi S, Omori T, Serikawa T and Hayashi M, Evaluation of the General suitability of the rat for the micronucleus assay : the effect of cyclophosphamide in 14 strains, Mutation Res., 495: 127-134, (2001).
96. Das UB, Mallick M, Debnath J and Ghosh D, Protective effect of ascorbic acid on cyclophosphamide- induced testicular gametogenic and androgenic disorders in male rats, Asian J Androl, 4:201-207, (2002).
97. Aidoo A, Lyn-Cook LE, Lensing S and Wamer W, Ascorbic acid (vitamin C) modulates the mutagenic effects produced by an alkylating agent *in vivo*, Environ Mol Mutag, 24: 220-228, (1994).