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Ascorbic Acid Prevents the Genetic Damage induced by Cyclophosphamide in *In vitro* Human Lymphocytes

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ABSTRACT

Cyclophosphamide is one of most commonly used in malignant lymphomas. The antioxidants such as Vit C inhibits the toxicity of mutagens/carcinogens in animals and Humans. The protective effects of ascorbic acid (AA) on cyclophosmide (CP) induced cytogenetic damage was evaluated suing analysis of chromosomal aberrations in human lymphocytes in vitro. When cultures were treated with various doses of ascorbic acid there was no increase in the frequency of chromosomal aberrations in human lymphocyte cell cultures at all dose levels. But when AA was added to CP significant decrease in the percentage of chromosomal aberrations was observed in human lymphocytes vitC protects the CP in induced genetic damage in human lymphocyte cultures. Hence the results clearly indicate AA supplementation is safer in chemotherapis regimen for treatment of cancer. The VitC protects the cyclophosphamide induced genotoxicity in germ cells of mice. Hence VitC supplementation is safer in chemotherapeutic strategy.

Key words: *Ascorbic acid, genotoxicity, cyclophosphamide, human lymphocytes.*

INTRODUCTION

Cyclophosphamide, Cisplatin, Tamoxifen, Gemcitabine and Paclitxel etc., have shown clastogenic effects in various test systems. Potential genetic damage due to drugs and other chemicals is well recognized. Extensive studies have been carried out on mutagenicity of various drugs in microorganisms, insects, mammals and in exposed population[2,33,46]. Cyclophosphamide (CPM) is a well-known bifunctional alkylating agent, widely used in cancer chemotherapy and expresses its genotoxicity when metabolically activated[9]. It is extensively used for the treatment of various cancers as well as an immunosuppressant in organ transplantation, rheumatoid arthritis, systemic lupus erythromatosis, multiple sclerosis, and other benign diseases[13,35]. According to the International Agency for Research on Cancer (IARC), CPM is widely used as reference mutagen and has been classified as carcinogenic for animals and humans[47].

There is considerable evidence that the effects of mutagenic and carcinogenic agents can beltered by many dietary constituents.

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Ascorbic acid (VC) is an essential dietary nutrient required as a co-factor for many enzymes and a very efficient antioxidant, scavenging reactive oxygen and nitrogen species and protecting cells against free radical mediated damage. Besides exerting antioxidant influence directly, VC can promote the removal of oxidative DNA damage from the DNA and/or nucleotide pool, through the up-regulation of repair enzymes[7]. The inhibitory effect of VC towards a number of mutagens/carcinogens was shown by many authors in humans and animals[14,22,28]. The goal of the present study is to evaluate the in vivo protective effect of VC against the DNA damage induced by cyclophosphamide in mouse somatic cells. While most animals synthesize their own vitamin C, humans and 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[21]. It is necessary in the body to form collagen in bones, cartilage, muscle and blood vessels and aids in the adsorption of iron[23]. Some genotoxic effects of vitamin C in invitro test systems has been demonstrated but in invivo experiments there are no genotoxic effects[5,16] in the present investgation analysis of chromomal abberations in humamn lymphocytes *in vitro*.

Dosage Schedule

In the present study two experiments were conducted. The animals were feed orally with cyclophosphamide and PFE extract and categorized in to following groups

Group I: controls with 0.5ml of physiological saline.

Group II: PFE extract 33 µg/ml

Group III: PFE extract 68 µg/ml

Group IV: PFE extract 114 µg/ml

In the second experiment for modulation studies all the three groups as follows:

Group I : controls with 0.5ml of physiological saline.

Group II: Cyclophosphamide 100 µg/ml

Group III: Cyclophosphamide 100 µg/ml + PFE extract 33 µg/ml

Group IV: Cyclophosphamide 100 µg/ml + PFE extract 68 µg/ml

Group V: Cyclophosphamide 100 µg/ml + PFE extract 114 µg/ml

Analysis of chromosomal aberrations in Human Lymphocytes

Intravenous blood of healthy human adults was collected aseptically using an anticoagulant heparin. Lymphocyte cultures were prepared and harvested according to the standard method prescribed by Moorhead *et al*[30] Lymphocyte cultures were initiated in RPMI 1640 medium containing 25% human AB serum, 0.5% PHA & 0.25% antibiotic Dicrysticin from Sigma Aldrich. The concentration of lead nitrate was150 µg/ml and the ascorbic concentrations were calculated based on therapeutic dose. The doses were 3.33µg/ml, 8.33µg/ml and 16.33µg/ml. For priming experiments 150+3.33, 150+8.33 and 150+16.33 µg/ml were added to the cell cultures and at an interval of 0, 24 & 48 hrs. Simultaneously the control group cultures were also maintained. The cultures were grown at 37^{o}C for 72 hrs. Later all cultures were terminated by adding colchicine (0.02%) to arrest the cell cycle at metaphase. After 2 h. of treatment the material was centrifuged at 1000 rpm for 10 min. The cells were treated with prewarmed hypotonic solution (KCI 0.08 M) for 20 min. After hypotonic treatment the cultures were centrifuged and the supernatant was removed, cells were fixed by adding chilled fixative (Methanol: Acetic acid 3:1). Later the slides were examined for various types of chromosomal aberrations such as chromatid gaps, breaks, acentric fragments. Isochromatid gaps, breaks and polyploids. Metaphases were scored per culture and the data was analyzed statistically using 2x2 contingency Chi-square test.

RESULTS

The results showed that the percentage of chromosomal aberration at 24 hrs treatments was increased from 2.00 in control to 2.25, 3.25 and 3.5 in cultures treated with 3.33, 8.33 and 16.66 g/ml ascorbic acid (Table 1). Similarly at 48 hrs the frequency of

chromosomal aberrations in cultures treated with 3.33, 8.33 and 16.66 g/ml ascorbic acid as 3.0, 3.25 and 3.75 as against 2.25 in control cultures. At 72 hrs treatment the frequencies of chromosomal aberrations were 1.5 in control cultures and it has increased to 4.0, 4.5 and 5.25 in 3.33, 8.33 and 16.66 g/ml ascorbic acid treated cultures. The overall incidences of frequencies were found to be statistically significant at all dose levels and all time intervals. (P>0.05, Table-1).

In the present study, the frequency of total chromosomal aberrations of Cyclophosphamide + Ascorbic Acid treated invitro human lymphocyte cultures were 0.80 in control 7.8 in 100 g/ml of cycophosphamide treated cultures where as a decrease to 4.8, 4.20 and 3.0 was observed in Cyclophosphamide + Ascorbic Acid primed cultures i.e. 100+3.33 g/ml, 100+8.33 g/ml and 100+16.66 g/ml respectively at 24 hrs exposure (Table 2). Similarly at 48hrs 1.40 in control and 7.40 at 100 g/ml cp exposure and decreased after priming with ascorbic acid as follows 5.2, 4.4 and 3.4 with 100+3.33 g/ml, 100+8.33 g/ml and 100+16.33 g/ml conc. respectively. At 72 hrs controls were 1.8 and cp treated values are 7.6 and after priming with ascorbic acid with 100+3.33, 100+8.33 and 100+16.66 were 5.40, 5.0 and 3.6 respectively (Table-2). The data was statistically analysed and found to be significant (Table –2).

Table 1: Frequency of Chromosomal aberrations recorded in invitro human peripheral lymphocytes with various doses of Ascorbic Acid for 24, 48 and 72 hrs interval

Dose (µg/ml) and duration of treatment (hr)	24hrs		48 hr		72 hr	
	Normal metaphases scored (%)	Abnormal metaphases scored (%)	Normal metaphases scored (%)	Abnormal metaphases scored (%)	Normal metaphases scored (%)	Abnormal metaphases scored (%)
Control	32	8	391	9	394	9
	(98.00)	(2.0)	(97.75)	(2.25)	(98.5)	(1.5)
3.33µg/ml	391	9	388	12	384	16
	(97.75)	(2.25)*	(97.0)	(3.0)*	(96.0)	(4.0)*
8.33µg/ml	387	13	387	13	382	18
	(96.75)	(3.25)*	(96.75)	(3.25)*	(95.5)	(4.5)*
16.66µg/ml	386	14	385	15	379	21
	(96.5)	(3.5)*	(96.25)	(3.75)*	(94.75)	(5.25)*

*P<0.05

The values in parenthesis are percentages

Table 2: Frequency of Chromosomal aberrations recorded in invitro human peripheral lymphocytes analysed after for 24, 48 and 72 hrs in cycophosphamide treated cultures primed with Ascorbic Acid

Dose Time	Non primed		Primed with ascorbic acid					
			3.33µg/ml		8.33µg/ml		16.66µg/ml	
	Normal metaphases scored (%)	Abnormal metaphases scored (%)	Normal metaphases scored (%)	Abnormal metaphases scored (%)	Normal metaphases scored (%)	Abnormal metaphases scored (%)	Normal metaphases scored (%)	Abnormal metaphases scored (%)
24 hours								
Control	492 (99.20)	8(0.80)						
Mitomycin	452 (90.40)	48 (9.60)*						
100 µg/ml	461 (92.20)	39 (7.80)*	476 (95.20)	24 (4.80)*	479 (95.80)	21 (4.20)*	485 (97.0)	15 (3.00)*
48 hours								
Control	493 (98.60)	7 (1.40)						
Mitomycin	450 (90.0)	50 (10.0)*						
100 µg/ml	463 (92.60)	37 (7.40)*	474 (94.80)	26 (5.20)*	478 (95.60)	22 (4.40)*	483 (96.60)	17 (3.40)*
72 hours								
Control	491 (98.2)	9(1.80)						
Mitomycin	451 (90.20)	49 (9.80)*						
100 µg/ml	462 (92.40)	38 (7.60)*	473 (94.60)	27 (5.40)*	485 (95.0)	25 (5.00)*	482 (96.40)	18 (3.60)*

*P<0.05

The values in parenthesis are percentages

DISCUSSION

Among the alkylating agents used for the treatment of wide range of cancers, CP is one of the widely used drugs. Acrolein and phosphoramide are the active compounds of CP. These active compounds of the CP slow down the growth of cancerous cells by interfering with the actions of DNA within those cells. The mutagenicity of CP in particular is related to formation of the ultimate cytotoxic metabolite phosphoramide mustard through the intermediate agentsine C. cause gene mutations, CA and rearrangements and aneuploidy in somatic cells as well as an increased frequency of secondary treatment-related tumors in human cancer survivors[6,36] Hence the development of effective modulatory hydroxycyclophosphamide and deschloroethylcyclophosphamide[27,39] which is capable of inducing DNA crosslinks and strand lesions[20] It has been tested extensively for its genotoxic effects both *in vitro* and in vivo in different test systems giving consistently positive results[18]. Several lines of studies have demonstrated that the CP and many other chemotherapeutic agents cause gene mutations, CA and rearrangements and aneuploidy in somatic cells as well as an increased frequency of secondary treatment-related tumors in human cancer survivors[6,36]. Hence the development of effective modulatory strategies for CP induced toxicity

will be of great importance for the chemotherapy for cancer. During the recent years much focus has been given for the search for natural compounds which modulates the drug/chemical induced toxicity[28,44,4548]. Several studies have clearly established the protective effects of various phytonutrients upon drug-induced toxicity[8,25,26,37,40.]

The present work is an attempt to study the modulatory effect of Ascorbic acid in cyclophosphamide induced DNA damage in *in vitro.* Vit. C acts as either a free radical scavenger or pre-oxidant producing hydrogen peroxide and free radicals[3,32,43]. As a physiological molecule ascorbic acid contributes to the natural antioxidant defense in cells Ascorbic Acid is considered to be most important antioxidant of plasma and at least in some cell types of the intracellular has ascorbate may be in mM range[19] AA is not genotoxic in tests *in vivo* may be due to spatial separation of living organisms of the components of oxy radical generating system (namely AA, transition metal ions and oxygen, which has a much lower partial pressure in tissues than in the atmosphere) and also due to existence of an antioxidant system that inactivates oxygen radicals before their interaction with DNA[15].

The results of the present study showed AA administration significantly decreases in chromosomal aberrations induced by lead nitrate in human lymphocytes *in vitro*. Numerous studies have showed antioxidant nature of vit C on DNA stand breakage in cell cultures[11,17,31,34] Vit C supplementation however did not show a measurable protective effect against DNA damage[4] and failed to protect the genotoxic damage caused by cisplatin based chemotherapy[12]. The result are comparable with that konopacka *et al*[24] who noted the antioxidant vit C reduced the DNA damage before and after gamma ray irradiation of human lymphocytes in *in vitro*. Vit C also decreases chromosomal damage in Humans[10] However Ahmed *et al*[1] reported that vit C reduce the frequency of chromosomal aberrations in peripheral blood lymphocyte cultures against hydrocortisone genotoxicity. Similar results were obtained with AA added cultures when treated by chromium in *in vitro*. Similarly, the percentage of chromosomal aberrations were decreased by AA in cadmium chloride induced genetic damage in *in vitro* lymphocytes[38].

Interest in the chemo preventive functions of antioxidants has grown considerably in recent years. Evidence accumulated over the year's shows that people with high dietary intakes of fruits and vegetables are less likely to develop cancer than people who have low dietary intake of these foods. While many chemopreventives in fruits and vegetables may have anticancer properties, much interest has focused on vitamin C[41]. This study represents one of the premiere studies carried out to diminish the toxicity and the genotoxicity of the oxidative compound TMT by using the natural antioxidant compound VC. Vitamin C is a highly effective antioxidant. It acts as a reducing agent that can terminate free radical driven oxidation by being converted to a resonance-stabilized free radical. In this respect VC can protect indispensable molecules in the body, such as protein, lipids, carbohydrates and nucleic acids (DNA and RNA). VC also regenerates other antioxidants such as vitamin E[41]. Our results showed that concurrent administration of VC inhibited the DNA damage and chromosome aberrations induced by cyclophosphamide in all tested doses. The antioxidant properties of ascorbic acid could be an important factor in the protection against CP induced DNA damages and tissue toxicity and ascorbic acid has been reported as an anti sister chromatid exchanges induced by cyclophosphamide in mice. Earlier also the protective role of vitamin C against the mutagenic effects of various chemical agents i.e., cisplatin[16] norethynodrel[45] and bleomycin[49] as well as cyclophosphamide[49] has been reported.

CONCLUSIONS

At this point of time more investigation is needed to delineate the down regulation pathways of modulatory actions of extract of

phyllanthus and which component of it is exerting effect on CP induced genotoxicity in human lymphocytes in *in vitro*. We here predict that this finding of ours give the directions for the future research possibilities for the design and development of plant extract related modulatory drugs in combination with the CP. Such drugs might minimize the side effects caused by the widely used chemotherapeutic agent CP.

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