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PROTECTIVE MEASURES OF VITAMIN C EXTRACT ON CISPLATIN-INDUCED CYTOGENOTOXICITY IN HUMAN LYMPHOCYTES *IN VITRO*

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ABSTRACT

Naturally occurring antioxidants have been extensively studied for their capacity to protect organisms and cells from oxidative damage. Many plant constituents including Vitamin C appear to be potent antimutagens and antioxidants. The present investigation was directed to study the possible modulatory effects of Vitamin C on chromosomal aberrations (CA) and sister chromatid exchanges (SCE) induced by Cisplatin were performed in the human lymphocyte cultures in vitro. Three different doses of Vitamin C were tested for their modulatory capacity on the mutagenecity exerted by Cisplatin. The results indicate that Vitamin C decreased significantly the percentage of chromosomal aberrations and sister chromatid exchanges induced by Cisplatin in a dose dependent manner indicates the modulatory effects of Vitamin C**.**

Keywords: *Cisplatin, Vitamin C, chromosomal aberrations, sister chromatid exchanges, human lymphocytes.*

Number of Tables: 2 **Number of References** : 39

INTRODUCTION

Platinum-derived drugs are playing an increasing important role in the treatment of a variety of neoplasms (Olivi *et al.*, 1993). Cisplatin, parent compound of this class, is one of the chemotherapeutic agents for cancer treatment. Cisplatin has been used to treat cancer for more than 30 years. The antitumor action of CP is atributted to its action on DNA synthesis . However, the use of CP is limited because of its toxicity to normal tissues (Rosenberg, 1985). The high mutagenic potency of CP raises the concern that its use in cancer chemotherapy may be responsible for secondary malignancies, which have been observed in animals and some cured patients treated with CP (Kempf and Ivankovic, 1986; Greene, 1992;). The generation of free radicals is believed to be an important mechanism in the development of cisplatin toxicity (Masuda *et al.*, 1994; Baliga *et al.*, 1998; Weijl *et al*, 1998; Wozniak *et al*, 2004). Much effort has been put into reducing the mutagenic side effects of cisplatin by administration of modulating agents, usually free radical scavenges. Free radical-mediated reactions are responsible for a wide range of chemotherapy-induced side effects, and antioxidants are able to protect non-malignant cells and organs against damage by cytostatic agents (Weijl *et al.*, 1997).

Recently a variety of compounds that possess antimutagenic properties has been detected in vegetables and spices whose dietary intake decreases the risk of cancer and other malignant diseases in human. Certain naturally occurring substances in plants and other sources have protective effects against environmental mutagens/ carcinogens and endogenous mutagens (Ferguson, 1994). It has been suggested that the common use of antimutagens and anticarcinogens in every days life will be most effective procedure for preventing human cancer and genetic disease. There are several reports on the antimutagenic and anticarcinogenic effect of food components like turmeric. Epidemiological studies proved the protective effects of antioxidants against cervical displasia as well as cancers.

Vitamin C (ascorbic acid) is a six-carbon lactone that is synthesized from glucose in the liver of most mammalian species, but not by humans, non-human primates and guinea pigs. These species do not have the enzyme gulonolactone oxidase, which is essential for synthesis of the ascorbic acid immediate precursor 2-keto-l-gulonolactone. The DNA encoding for gulonolactone oxidase has undergone substantial mutation, resulting in the absence of a functional enzyme (Nishikimi M et al.,1994, Nishikimi M et al.,1996). vitamin C can be oxidized by many species that have potential to be involved in human diseases (Halliwell B, 1999 ;Neuzil et al.,1997). Vitamin C is considered to be one of the most prevalent antioxidative components of fruit and vegetables, and it could exert chemo preventive effects without apparent toxicity. It has also been used as a dietary supplementintended to prevent oxidative stress–mediated chronic diseases such as cancer, cardiovascular disease (Khaw K.T., 2001), hypertension (Duffy S.J., 1999), stroke (Kurl S., 2002), and neurodegenerative disorder (EngelhartM.J., 2002). Although it has

generally been acknowledged that vitamin C protects cells from oxidative DNA damage, thereby blocking the initiation of carcinogenesis, some studies have shown that dietary vitamin C supplementation is not beneficial but, rather, may cause DNA damage. chemo preventive mechanism of vitamin C may be linked to the inhibition of other processes- in particular, tumor promotion- rather than to that of tumor initiation. Now-a-days natural products of plants are being treated as replacement of synthetic agents in nearly all filed of medicine. Antimicrobial and antifungal agents are also a part of this. The demand of natural food additives is increasing day and day. Natural products from plants can be a better option for the replacement of synthetic antimicrobial and chemo preventive agents. Hence the present study is designed to evaluate the protective effects of Vitamin C extract on Cisplatin induced genotoxicity in the human lymphocyte cultures *in vitro*.

MATERIAL AND METHODS

Chemicals: RPMI 1640 medium, new born fetal calf serum and phytohaemagglutinin – M were purchased from Gibco. 5 – Bromo – 2 – deoxyuridine and Hoechst 33258 stain (40 Dg ml-1) from Sigma Aldrich, Colchicine from Loba-Chemie, 3% Giemsa stain solution in phosphate buffer (pH 6.8) from E. Merck, India, Methanol (SRL chemicals), Cisplatin from Cipla India were obtained. All other chemicals used were of analytical grade.

Preparation of Vitamin C Extract

Fresh Vitamin C were purchased from the local market and made into coarse powder with mortar and pestle. The powder (about 250g) was soaked in 500ml of ethanol for 72 hours. The solvent was runned through rotavapour to separate solvent and concentrated through Soxhlet apparatus. The obtained Vitamin C alcoholic extract was runned through rotavapour for further concentration. Final extract was lyophilized to powder form and stored at 4°C until use (Mara *et al.*, 2003).

Human lymphocyte culture

The heparinized (100 units/ml) blood samples were obtained from healthy donors under aseptic conditions, with no recent history of exposure to mutagens. For each culture, the blood samples (0.5 ml) was placed in a sterile culture vial containing 5 ml of RPMI 1640 medium supplemented with 1 ml of fetal calf serum, antibiotics, 0.1 ml of phytohaemagglutinin and incubated at 37^{o}C. At 70h after initiation, all cultures were terminated by the addition of colchcine (0.05% to each vial) to arrest the cell cycle at metaphase. After 2h of colchicine treatment the cultures were harvested according to the standard method described by Moorhead *et al* (1960). Control cultures were maintained simultaneously. Various doses of Vitamin C (0.12, 0.24, 0.48 mg/ml) and 0.12 mg/ml of Cisplatin were added separately to the culture vials. For modulation antimutagen was added at the initiation of the culture and Cisplatin was added on 2nd day. Cultures were harvested on 5th day, which corresponds to 72hours exposure to Cisplatin respectively. Each sample was

maintained in quadrates. The normal and positive controls were also maintained simultaneously.

Chromosomal aberrations analysis

Cells were centrifuged at 1000 rpm for 10 min. The supernatant was removed and 5 ml of pre-warmed (37^{o}C) 0.075 M KCl hypotonic solution was added to the cultures and incubated at 37^{o}C 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). The slides were prepared by air drying method method described by Moorhead *et al.*, (1960) and stained with giemsa (2%) stain for 20 min. Later the slides were screened for chromosomal aberrations. For each concentration of treated and control groups 400 metaphases were scored.

Sister chromatid exchanges analysis

For SCE analysis, 5- Bromo-2-deoxyuridine (3 µg ml-1) was added at the beginning of the initiation of the culture. Mitotic arrest was done two hours prior to harvesting by adding 0.05% of colchicine. Hypotonic treatment and fixation were done in the same way described for CAs. The slides were processed according to the method described earlier by Perry and Wolff (1974). For each concentration 30 metaphases were scored for SCE in control and treated groups.

Microscopic Examination

Slides were prepared from all the cultures of different doses. The slides were stained with 2% giemsa (2ml of 2% giemsa and 2ml of phosphate buffer added to 46 ml 0f DDW). Air dried slides were coded and screened for the presence of various types of chromosomal aberrations and sister chromatid exchanges. 400 well spread; non overlapping metaphases were studied per concentration.

Statistical analysis

The data on CAs were analyzed statistically using 2x2 contingency Chi – Square test. Student's t – test was used for calculating the statistical significance in SCE.

RESULTS AND DISCUSSION

The results on the incidence of CAs in *in vitro* lymphocytes after treatment with CP and various doses of Vitamin C extract individually and CP primed with Vitamin C are shown in the Table 1 and for SCEs the results were depicted in Table 2.The suitable method adopted for studying cytogenetic effects induced by a suspected agent in human beings is the micro culturing of human peripheral blood lymphocytes. It is an essentially important and sensitive indicator for both *in vivo* and *in vitro* induced structural and numerical aberrations. As expected, cultures treated with CP (0.12 mg/ ml) had a higher frequency of CAs than the controls and dose dependent decrease was observed when treated with Vitamin C extract. Maximum decrease in CAs was found at the higher dose of Vitamin C extract rather than with the lower doses. The decrease in CAs was statistically significant ($p<0.05$) for 0.12, 0.24, 0.48 mg/ml of Vitamin C extract primed groups in comparison to CP alone treated lymphocytes. For the Vitamin C extract alone treated group, the total no of aberrations did not show any significant level of changes. For the CP alone treatment, the percentage of aberrations scored 17.25 which decreased to 12.00, 9.00, 7.00 in Vitamin C primed

cultures. This decrease in CAs was statistically significant. The CAs is one of the widely used parameters for testing the protective effects of natural compounds on the drug and chemical induced toxicity. The modulatory effect of natural compounds on the CAs induced by various kinds of chemicals and drugs is well established (Shukla and Taneja, 2002; Bhattacharya *et al.,* 2004; Siddique and Afzal, 2005a; Dutta *et al.,* 2007).Sister chromatid exchanges (SCEs) are symmetrical exchanges between newly replicated chromatids and their sisters. While homologous recombination may be one of the principal mechanisms responsible for SCEs. The SCEs are also used as one of the parameters for testing the protective effects of natural compounds on drug and chemical induced genotoxicity. In our experiments, the Vitamin C extract alone showed a dose dependent increase in the SCEs. There was increase in mean SCEs per cell when the concentration of Vitamin C extract increased from 0.12 to 0.48 mg/ml. But such mean increase was not statistically significant. On the contrarary, in the priming experiments, when the cells were treated with both CP and Vitamin C, there was a significant decrease in SCEs was observed. For the CP alone group, the mean SCE per cell was scored 7.33 which decreased to 3.17 when primed with Vitamin C extract. Vitamin C was found to be non-mutagenic in mice, rats, in cultured mammalian cells (Vijayalaxmi, 1980; Kuttan *et al.,* 1985) and in the Ames test (Nagabhushan *et al.,* 1987). Several studies have clearly established the protective effects of various phytonutrients upon drug-induced toxicity (Darroudi *et al.,* 1988; Kumaraguruparan *et al.,* 2005; Kumar and Kuttan, 2005;, Cisplatin (Antunes *et al.,* 2000), cyclophosphamide and mitomycin C (Abraham, 1996), cyclophosphamide (Shukla *et al.,* 2003), chemicals like benzo[a]pyrene (Polasa *et al.,* 2004; Azuine and Bhide, 1992; Shukla *et al.,* 2003), hydrocortisone (Sultan Ahmad *et al.,* 2004), 7,12-dimethylbenz[a]anthracene (Azuine and Bhide 1992;Chandramohan et al.,2004); radiation (Srinivasan *et al.*,2006, Adhvaryu *et al.,* 2008) in *in vitro* and *in vivo* mammalian test systems. The results of present investigation conclude a dose dependent antigenotoxic potential of Vitamin C against cisplatin induced genotoxicity in *invitro* human lymphocytes. However, the mechanism by which Vitamin C acts remains to be investigated, and further studies are necessary to clarify this point.

Table 1: *Effect of different concentrations of Vitamin C extract on CAs induced by Cisplatin in the cultured human lymphocytes*

Treatment Percentage of aberrations	**Chromatid aberrations**				**Isochromatid aberrations**		**Number of Polyploidy cells**
	Gaps	**Breaks**	**Acentric**	**Fragments**	**Gaps**	**Breaks**	
Control	3.50	6	4	2	1	1	0
CP (0.12 mg/ml)	17.25*	22	16	11	8	7	5
Vitamin C Extract							
0.012mg/ml	4.50	7	6	2	2	1	0
0.24 mg/ml	5.25	7	6	4	2	1	1
0.48 mg/ml	5.75	8	6	4	2	2	1
Vitamin C + CP							
0.012 + CP	12.00*	14	11	9	7	4	3
0.24 + CP	9.00*	12	9	6	4	3	2
0.48 + CP	7.00*	10	7	4	3	2	2

*Significant at *P < 0.05 level*

Table 2: *Effect of Vitamin C on SCEs induced by CP in the cultured human lymphocytes*

Treatment	**No of metaphases scored**	**Total No. of SCE's scored**	**Mean SCE's per cell**
Control	30	15	0.50
CP (0.12 mg/ml)	30	225*	7.33
Vitamin C Extract			
0.012 mg/ml	30	19	0.63
0.24 mg/ml	30	23	0.77
0.48 mg/ml	30	26	0.87
Vitamin C + CP			
0.012 + CP	30	75*	2.50
0.24+ CP	30	88*	2.93
0.48+ CP	30	95*	3.17

**P<0.05*

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