

Modulatory effects of garlic extract against the cyclophosphamide induced genotoxicity in human lymphocytes *in vitro*

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Abstract: Cyclophosphamide (CP) is a commonly used chemotherapeutic and immunosuppressive agent which is used in the treatment of wide range of cancers and autoimmune diseases. Besides that it is a well known carcinogen. In this study by using chromosomal aberrations (CA) and sister chromatid exchanges (SCE) assays method, the modulatory effects exerted by the extract of garlic against the CP induced genotoxicity in the human lymphocyte cultures *in vitro* were tested. Three different doses of garlic extract were tested for their modulatory capacity on the mutagenicity exerted by 100 µg ml⁻¹ of CP. The results indicate a significant decrease in the frequency of CA and SCE suggesting that the garlic extract modulates the CP induced genotoxicity in a dose dependent manner. These findings provide the future directions for the research on design and development of possible modulatory drugs containing garlic extract.

Key words: Cyclophosphamide, Garlic extract, Genotoxicity, Chromosomal aberrations, Sister chromatid exchanges, Human lymphocytes
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Introduction

For centuries, the garlic is used as a medicinal herb. It is having wide range of medicinally important properties like, antimutagenic, anticancer, anti-inflammatory, antihypertensive, antimicrobial, antifungal, antidote, hepatoprotective, hyperglycemic, immunomodulation etc. (Agarwal, 1996; Banerjee *et al.*, 2003; Khanum *et al.*, 2004). Several studies in the recent years have shown the antigenotoxic and antimutagenic effects of garlic for various drugs and chemicals (Shukla and Taneja, 2002; Bhuvanawari *et al.*, 2004; Siddique and Afzal, 2005a; Belloir *et al.*, 2006). Studies of the anticarcinogenic effects of garlic on several carcinogens were found to be effective in different ways such as direct inhibition of tumor cell metabolism, inhibition of initiation and promotion phases of carcinogenesis and modulating the post immune response and besides all these garlic acts as a strong antioxidant by its ability to scavenge free radicals, (Wei and Lau, 1998). Sulfur rich constituents of garlic such as diallyl sulfide (DAS) and diallyl disulfide (DADS) are known to induce activities of phase II enzymes, which in turn reduce the genotoxicity of several carcinogens (Guyonet *et al.*, 2002).

Cyclophosphamide is a commonly used chemotherapeutic and immunosuppressive agent for the treatment of a wide range of neoplastic as well as some autoimmune diseases. With increased success rate of cancer treatment, due in part to the aggressive use of high combination drug therapies, there has been growing concern about the long term side effects (carcinogenic) of these alkylating agents and other neoplastic drugs. There are several reports indicating the carcinogenic effects of CP in humans and animals (Ember *et al.*, 1995; Ridder *et al.*, 1998).

In this study by using chromosomal aberration (CA) and sister chromatid exchange (SCE) assays method, we have analyzed the modulatory effects exerted by the garlic extract against the cyclophosphamide induced genotoxicity in the human lymphocyte cultures. The CA test using cultured mammalian cells is one of the sensitive methods to predict environmental mutagens and/or carcinogens. SCE technique is considered as an important method for the cytogenetic mutagenicity testing. Even though SCE is generally more sensitive indicator of genotoxic effects than structural aberrations (Tucker and Preston, 1996), which however is not adequate to replace classical methods of analysis of structural chromosomal aberrations. Both CA and SCE assays methods are widely used as the parameters for testing the modulatory effects of natural compounds on drug and chemical induced genotoxicity.

Materials 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 µg ml⁻¹) from Sigma Aldrich, Colchicine from Loba-Chemie, 3% Giemsa stain solution in phosphate buffer (pH 6.8) from E. Merck, India, cyclophosphamide (cyclohexan) from Biochem India were obtained. The garlic extract containing 2% allicin was purchased from Unicorn natural products. All other chemicals used were of analytical grade.

Human lymphocyte culture: Peripheral blood cultures were prepared in duplicate according to the method described earlier by Moorhead *et al.* (1960). The heparinized blood samples were obtained from healthy donors (N = 2) with no recent history of exposure to mutagens. For each culture, the blood samples

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(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 and 0.1 ml of phytohaemagglutinin and incubated at 37°C.

Chromosomal aberrations analysis: For CA analysis, after 24 hr, CP (100 µg ml⁻¹) and garlic extract were added in the concentration of 3, 6 and 12 mg/culture individually and in combinations with CP were kept for additional 48 hr of incubation. The normal and negative controls were also kept. The colchicine (0.2 ml) was added to each culture vial an hour before harvesting. Cells were centrifuged at 1000 rpm for 10 min. The supernatant was removed and 5 ml of pre-warmed (37°C) 0.075 M KCl hypotonic solution was added. Cells were resuspended and incubated at 37°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 (Rothfels and Siminovitch, 1938) and stained with giemsa (3%) stain for 20 min (Moorhead *et al.*, 1960). Later the slides were screened for chromosomal aberrations according to the standard protocol of Savage (1979). For each concentration of treated and control groups 100 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. After 24 hr CP (100 µg ml⁻¹) and garlic extract (3, 6 and 12 mg/culture) were added individually and in combinations with CP were kept for another 48 hr of incubation. Mitotic arrest was done an hour prior to harvesting by adding 0.2ml of colchicine. Hypotonic treatment and fixation were done in the same way described for CA. The slides were processed according to the method described earlier by Perry and Wolff (1974). For each concentration 50 metaphases were scored for SCE in control and treated groups.

Statistical analysis: The data on CA was analyzed statistically using 2x2 contingency Chi – Square test. Student's t – test was used for calculating the statistical significance in SCE and the level of significance were tested from standard statistical Tables of Fisher (1963).

Results and Discussion

The results on the incidence of CA in *in vitro* lymphocytes after treatment with CP and various doses of garlic extract individually and CP primed with garlic are shown in the Table 1.

The results indicate the induction of CA by CP and dose dependent decrease when treated with garlic extract. Maximum decrease in CA was found at the higher dose of garlic extract rather than with the lower doses. The decrease in CA was statistically significant ($p < 0.05$) for 3, 6 as well as 12 mg/culture garlic extract and CP treated groups in comparison to CP alone treated lymphocytes. For the garlic extract alone treated group, the total no of aberrations did not show any significance level of changes. For the CP alone treatment, the total number of aberrations scored

14.00 which decreased to 3.0 when treated with both CP (100 µg ml⁻¹) and garlic extract (12 mg/culture). This decrease in CA was statistically significant. The CA 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 CA 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).

The SCE are also used as one of the parameters for testing the protective effects of natural compounds on drug and chemical induced genotoxicity. In a recent study this parameter has been used for testing modulatory effects of allicin and L – ascorbic acid on the chlormadinone acetate induced genotoxicity (Siddique and Afzal, 2005b). In our experiments, the garlic extract alone showed a dose dependent increase in the SCE. There was increase in mean SCE per cell when the concentration of garlic extract increased from 3 to 12 mg/culture. But such mean increase was not statistically significant. On the contrary, in the priming experiments, when the cells were treated with both CP and garlic extract; there was a significant decrease in SCE. For the CP alone group, the mean SCE per cell was scored 10.34 which decreased to 4.72 when treated with both garlic extract (12 mg/culture) and CP (100 µg ml⁻¹). Such decrease was analyzed statistically using Student's t – test and found to be significant (Table 2).

Our results clearly indicate that garlic extract modulates the CP induced genotoxicity in a dose dependent manner in human lymphocytes *in vitro*. Also our results are comparable with the earlier published works where the garlic extract has showed a significant decrease in the CA and SCE induced by various chemicals and drugs (Bhattacharya *et al.*, 2004; Siddique and Afzal, 2005a,b; Yadav *et al.*, 2006). Chemoprotective effects of garlic extract against CP toxicity were reported earlier in animal models (Bianchini and Vainio, 2001; Shukla and Taneja, 2002; Premkumar *et al.*, 2004). The protective and modulatory effects of the garlic extract against the DNA damage, carcinogen bioactivation, and DNA adduct formation induced by the other compounds have been reported earlier on different cell lines (Belloir *et al.*, 2006; Milner, 2001; Hageman *et al.*, 1997).

Among the alkylating agents used for the treatment of wide range of cancers, CP is one of the widely used drugs. Acrolein and phosphoramidate 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 phosphoramidate mustard through the intermediate agents hydroxycyclophosphamide and deschloroethylcyclophosphamide (Le Blanc and Waxman, 1990; Ren *et al.*, 1997) which is capable of inducing DNA crosslinks and strand lesions (Hengstler *et al.*, 1997). It has been tested extensively for its genotoxic effects both *in vitro* and *in vivo* in different test systems giving consistently positive results (Hartmann *et al.*, 1995). Several lines of studies have demonstrated that the CP and many other chemotherapeutic agents

Table - 1: Effect of different concentrations of garlic extract on CA induced by CP in the cultured human lymphocytes

Treatment	Chromatid aberrations			Isochromatid aberrations		Total number of aberrations	Number of Polyploidy cells
	Gaps	Breaks	Acentric fragments	Gaps	Breaks		
Control	1.00	1.00	0.00	1.00	0.00	1.00	0.00
CP(100 µg ml ⁻¹)	7.00	7.00	2.00	3.00	3.00	14.00*	2.00
Garlic Extract							
3 mg/culture	1.00	1.00	0.00	0.00	0.00	1.00	0.00
6 mg/culture	1.00	1.00	0.00	1.00	0.00	1.00	0.00
12 mg/culture	2.00	1.00	1.00	1.00	0.00	2.00	0.00
Garlic + CP							
3+CP	5.00	5.00	1.00	1.00	2.00	9.00*	1.00
6+CP	4.00	3.00	1.00	1.00	1.00	5.00*	0.00
12+CP	3.00	2.00	0.00	0.00	1.00	3.00*	0.00

* Significant at p<0.05 level

Table - 2: Effect of garlic extract on SCE induced by CP in the cultured human lymphocytes

Treatment	No of metaphases scored	Total No. of SCE's scored	Mean SCE's per cell	Mean SCE's per chromosome
Control	50	89	1.78	0.0387
CP(100 µg ml ⁻¹)	50	517	10.34*	0.2248
Garlic extract				
3 mg/culture	50	107	2.14	0.0465
6 mg/culture	50	129	2.58	0.0561
12 mg/culture	50	147	2.94	0.0639
Garlic + CP				
3+CP	50	419	8.38*	0.1822
6+CP	50	312	6.24*	0.1356
12+CP	50	236	4.72*	0.1026

* Significant at p<0.05 level

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 (Povirk and Shuker, 1994; Ben Yehuda *et al.*, 1996). 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 (Unnikrishnan *et al.*, 1990; Shukla and Taneja, 2002; Siddique and Afzal, 2005b; Madhavi *et al.*, 2007). Several studies have clearly established the protective effects of various phytonutrients upon drug-induced toxicity (Darroudi *et al.*, 1988; Premkumar *et al.*, 2004; Kumaraguruparan *et al.*, 2005; Kumar and Kuttan, 2005). Experimentally, garlic and its associated sulfur components are reported to suppress the tumor incidence in breast, colon, skin, uterine, esophagus and lung cancers. This protection is presumed to arise from several mechanisms including enhanced DNA repair (Milner, 2001).

In this study we used the garlic extract containing 2% allicin. Among the major components of garlic, allicin, DAS, S-allylcystine and other organo-sulfur compounds have the radical scavenging activity and protective effects against chemical induced toxicity (Ciplea

and Richter, 1988; Thomson and Ali, 2003). Carcinogen induced DNA adducts are believed to be the initial step in carcinogenesis by chemicals, garlic extract showed a significant inhibition on DNA adduct formation (Hageman *et al.*, 1997). The DAS and related compounds from garlic have inhibitory effects on chemical carcinogenesis and mutagenesis (Yang *et al.*, 2001). The modulatory response of garlic may be attributed to the action of free radical scavenging, increasing the activity of antioxidant enzymes and inhibit the DNA adducts formation influencing the repair mechanism and modulating several metabolizing enzymes like cytochrome p450 and GST's (Bianchini and Vainio, 2001; Khanum *et al.*, 2004).

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