Antigenotoxic effect of lipoic acid against mitomycin-C in human lymphocyte cultures

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Abstract Antitumor agents are used in therapy against many forms of human cancer. One of these is mitomycin-C (MMC). As with many agents, it can interact with biological molecules and can induce genetic hazards in non-tumor cells. One of the possible approaches to protect DNA from this damage is to supply antioxidants that can remove free radicals produced by antitumor agents. Lipoic acid (LA) is known as one of the most powerful antioxidants. The aim of this study was to investigate antigenotoxic effects of LA against MMC induced chromosomal aberrations (CA), sister chromatid exchanges (SCE) and micronucleus (MN) formation in human lymphocytes. Lymphocytes were treated with 0.2 µg MMC/heparinized mL for 48 h. Three different concentrations (0.5, 1, 2 µg/mL) of LA were used together with MMC in three different applications; 1 h pre-treatment, simultaneous treatment and 1 h post-treatment. A negative, a positive

and a solvent control were also included. In all the cultures treated with MMC + LA, the frequency of abnormal cells and CA/cell significantly decreased compared to MMC. Statistically significant reduction was also observed in SCE/cell and MN frequencies in all treatments. These results demonstrated anticlastogenic and antimutagenic effects of LA against MMC induced genotoxicity. LA showed the most efficient effect during 1 h pretreatment. On the other hand, MMC + LA treatments induced significant reduction in mitotic index than that of MMC treatment alone. These results are encouraging that LA can be a possible chemopreventive agent in tumorigenesis in both cancer patients and in health care persons handling anti-cancer drugs.

Keywords Lipoic acid · Mitomycin-C (MMC) · Antigenotoxic effect · Chromosomal aberrations · Sister chromatid exchanges · Micronucleus

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Introduction

There is an increasing evidence that mutations in somatic cells are not only involved in the carcinogenesis process but also play a role in the pathogenesis of other chronic degenerative diseases, such as vascular, cardiovascular and cerebrovascular ones. These diseases are the leading causes of death in human populations (De Flora et al. 1996, 2001; De Flora and Ferguson 2005; Weakley et al. 2010). On the other hand, there is increasing evidence that cancer and other mutation-related diseases can be

prevented not only by avoiding exposures to recognized risk factors but also by intake of protective factors and modulating the defense mechanisms of the host organism. This strategy, referred to as chemoprevention, can be pursued either by means of suitable pharmacological agents and/or by dietary factors. In European countries and in the USA, mortality for cancer (De Flora and Ferguson 2005) has decreased during the last 15–20 years (Cimons 1988; Levi et al. 2002; De Flora et al. 2005; De Flora and Ferguson 2005; Jemal et al. 2010). These patterns indicate that cancer might be prevented.

Although it is not possible to make a generalization due to the multitude of diverse conditions involved, various chronic diseases may share common pathogenetic mechanisms such as DNA damage, oxidative stress, and chronic inflammation (De Flora et al. 1996; Lonkar and Dedon 2011). In addition, these diseases may share common risk and protective factors. These assumptions imply that certain prevention strategies applicable to cancer prevention are also beneficial to prevent other chronic diseases. On the other hand, the dietary regimen proposed for the prevention of cardiovascular diseases is basically the same for cancer prevention (De Flora and Ferguson 2005; Lee and Na 2010; Duthie 2011).

Antitumor agents and ionizing radiation are common therapy against many forms of human cancer (Gentile et al. 1998; Aziz et al. 2012). However, they can interact with specific biological molecules and genetic damages can be induced in non-tumor cells. Such damages may induce secondary tumors in cancer patients. Treatment with ionizing radiation and antitumor drugs from different categories lead to generation of free radicals in non-tumor cells both *in vitro* and *in vivo* (Weijl et al. 1997; Hernandez-Ceruelos et al. 2002; Davis et al. 2009; Lee and Na 2010). Mitomycin C (MMC) is one of these agents and known as an important antitumor drug and antibiotic. It has an extraordinary ability to crosslink the DNA with high efficiency (Szybalski and Iyer 1964; Tomasz 1995; Scarpato et al. 2008; Deans and West 2011). MMC is a bifunctional alkylating agent and causes genotoxic damage by producing hydroxyl radicals (Crooke and Bradner 1976; Krishna et al. 1986; Tomasz 1995; Bounias et al. 1997; Turkez et al. 2012). This damage, if not correctly repaired, can lead to genomic instability and increase the risk of developing cancer. DNA damages include single-strand breaks (SSBs),

double-strand breaks (DSBs), alkaline labile sites and various species of oxidized purines and pyrimidines (Selvakumar et al. 2006b; Shay et al. 2009).

Possible approach to protect DNA from damage is to supply antioxidants and to increase the capacity of cells to cope with DNA damage (Garcia et al. 2006; Lee and Na 2010; Tian et al. 2012). When antioxidants are applied to the cells with DNA damaging agents, antioxidants can remove free radicals before they damage the macromolecules such as DNA, proteins, and lipids (Bonorden and Pariza 1994; Surh 2003; Lee et al. 2011, Tian et al. 2012). Lipoic acid (LA) and its reduced form dihydrolipoic acid (DHLA) act as an antioxidant and reduce the clastogenic effects of antitumor agents (Nichols et al. 1997; Prahalathan et al. 2006; Selvakumar et al. 2006a, b; Lee and Na 2010). It was reported in the literature that LA significantly decreased the level of hydroxyl radicals, ROS, nitric oxide and lipid peroxidation in irradiated mouse skin fibroblasts. It was also reported that LA treatment reduced cell injury and protected cells against irradiation-induced cytotoxicity (Davis et al. 2009; Goraca et al. 2011). It is known that LA has a number of potentially beneficial effects in both prevention and treatment of oxygen-related diseases such as diabetes, atherosclerosis and heart diseases, cataract, neurodegenerative diseases, liver diseases, and cancer (Bilska and Wlodek 2005; Goraca et al. 2011; Tian et al. 2012).

Lipoic acid (chemical name: 1,2-dithiolane-3 pentanoic acid) or thioctic acid is found in virtually all prokaryotic and eukaryotic cells (Teichert et al. 2005; Lee and Na 2010). LA is a dithiol antioxidant found in mitochondria as the coenzyme for the pyruvate dehydrogenase and α -ketoglutarate dehydrogenase reactions (Davis et al. 2009; Tian et al. 2012). LA has been proven to be a natural, very powerful free radical scavenger, and antioxidant (Lee and Na 2010; Tian et al. 2012). In addition, LA inhibits the expression of a variety of inflammatory proteins and adhesion molecules (Zhang and Frei 2001; Bilska and Wlodek 2005). Antioxidant properties of both LA and its reduced form DHLA (Moini et al. 2002; Ho et al. 2007; Lee and Na 2010) and their protective effects against some antineoplastic drugs such as cyclophosphamide (Selvakumar et al. 2006a, 2006b) and adriamycin (Prahalathan et al. 2006) have been well documented in various *in vitro* and *in vivo* studies (Packer et al. 1995; Biewenga et al. 1997; Bustamante

et al. 1998; Drisko et al. 2003). Antioxidants act as biological response modifiers and are able to directly induce apoptosis in already established neoplastic cells. There is also supportive evidence that antioxidants enhance antitumor effects of chemotherapy (for references, see Drisko et al.'s review 2003) and induce apoptotic cell death in cancer cells but not in normal cells (Ahmad et al. 1997; Clement et al. 1998; Lee and Na 2010; Tian et al. 2012). The prevailing opinion is that the prooxidant action might be an important mechanism in anticancer and apoptosis inducing properties of phytochemicals (Azam et al. 2004; Ho et al. 2007; Lee and Na 2010; Tian et al. 2012). Because of these advantages, usage rate of LA has increased as a potential therapeutic agent (Scott et al. 1994; Cremer et al. 2006; Prahalathan et al. 2006; Shay et al. 2009; Goraca et al. 2011; Xiao et al. 2012). In addition to their antioxidant action, LA and DHLA, similar to vitamin E and vitamin C, potentially act as a pro-oxidant by reducing Fe(III) to Fe(II). Thus, they enhance liposome oxidation and hydroxyl radical formation (Biewenga et al. 1997; Pack et al. 2001; Ho et al. 2007; Lee and Na 2010). LA and DHLA act as a redox couple and their functions as either anti- or pro-oxidant, at least in part, are determined by the type of oxidant stress and the physiological circumstances (Packer et al. 1995; Biewenga et al. 1997; Çakatay 2006; Lee and Na 2010).

In general, two categories of LA containing foods have been identified. One is green plants such as broccoli, spinach and other green leafy vegetables which have a large amount of chloroplasts that are key spots for energy production in plants and require LA for this activity. Animal source foods constitute the second category of lipoic acid. Mitochondria are critical energy production spots in animal as well as plant cells, and the main location for finding lipoic acid. Body tissues with lots of mitochondria (like heart, liver, kidney, and skeletal muscle) are good spots for finding lipoic acid. Yeast has also been shown to contain this vital nutrient (Lodge et al. 1997; Akiba et al. 1998; Lee and Na 2010; Tian et al. 2012). Though available from these normal nutritional sources, it is not likely that appreciable amounts of LA have been consumed in the typical western diet. Dietary supplements that typically range from 50 to 600 mg are the primary sources of LA, and most information on its bioavailability comes from studies using supplements. According to these studies, LA bioavailability may be

dependent on multiple carrier proteins. Identification of such a multifaceted uptake and tissue distribution system suggests that various factors such as substrate competition, transcriptional, translational, and post-translational regulatory mechanism could influence the overall LA absorption (Shay et al. 2009). Due to its multimodal means of transportation, gastrointestinal absorption of LA appears to be quite variable. For example, Teichert et al. (1998) measured the plasma LA level in 200 mg R,S-LA (racemic mixture obtained by the manufacturing process; α -lipoic acid has one chiral center and therefore exists in both R- and S-enantiomeric forms) given volunteers and observed that approximately 20–40 % of R,S-LA was absorbed.

This study was undertaken to assess the protective effect of LA in human lymphocytes treated with MMC, an antitumor agent, on the basis of chromosomal aberrations, sister chromatid exchanges, and micronuclei frequency. MMC is used against solid cancer but has adverse effects in bone marrow and lymphoid tissues, and increase risk of leukaemia and lymphomas.

Materials and methods

Chemicals

Lipoic acid (CAS no 1077-28-7) was obtained from Applichem, mitomycin-C (CAS no 50-07-7), cytochalasin-B (CAS no 14930-96-2), bromodeoxyuridine (CAS no 59-14-3) and DMSO (CAS no 67-68-5) were obtained from Sigma. Lipoic acid (purity 98 %) was dissolved in ethanol (50 %), Cytochalasin-B was dissolved in DMSO (%99) and the other chemicals were dissolved in distilled water.

Cell cultures and treatments

Human peripheral blood samples were obtained from two healthy, non-smoking donors, one male and one female, aged 24–25 years. The heparinized blood (0.2 mL) was added to 2.5 mL chromosome medium containing fetal bovine serum, heparin, antibiotics and phytohaemagglutinin (PHA) (Biochrom) and incubated for 72 h at 37 °C. Three different concentrations of LA (0.5, 1, 2 μ g/mL) were used. Test concentrations were chosen on the basis of literature data and preliminary tests. All the cultures were treated with 0.2 μ g/mL MMC for the last 48 h. For each

concentration of LA, three cultures were established; 1 h pre-treatment, simultaneous treatment and 1 h post-treatment. For simultaneous treatment, MMC and LA were added together to the cultures. LA was added to the cultures 1 h prior to MMC as pre-treatment and 1 h after MMC as post-treatment. Treatment with MMC as positive control and ethanol as solvent control and an untreated control were also maintained.

Chromosomal aberrations (CA) and sister-chromatid exchange (SCE) assay

For the CA and SCE assays, whole blood was added to chromosome medium supplemented with 10 µg/mL bromodeoxyuridine. The cultures were incubated in the dark at 37 °C for 72 h. Colchicine was added at a concentration of 0.06 µg/mL for the last 2 h of the culture. The cells were harvested by centrifugation (923×g, 10 min), resuspended in hypotonic KCl (0.075 M) for 30 min at 37 °C and fixed in a mixture of cold methanol: acetic acid (3:1) for 45 min at +4 °C. Cells were fixed for 2 more times and finally, metaphase spreads were prepared by dropping the cell suspension onto slides. For chromosome aberrations, air-dried slides were stained with 5 % Giemsa (pH 6.8) in Sorensen buffer for 15–20 min, and then washed in distilled water, dried at room temperature and mounted with depex. For the SCE assay, slides were stained with Giemsa according to Speit and Houptner (1985) with some modifications. For the CA assay, one hundred well-spread metaphases were analyzed per donor (totally 200 metaphases per concentration). For the SCE assay, a total of 50 cells (25 cells from each donor) under second mitoses were scored for each experimental concentration. In addition, a total of 200 cells (100 cells from each donor) were scored for the determination of replication index (RI) calculated according to the following formula: $RI = (M_1 + 2M_2 + 3M_3)/N$, where, M_1 , M_2 and M_3 represent the number of cells undergoing first, second and third mitosis and N is the total number of metaphase scored. 1,000 cells from each donor were analyzed to obtain the mitotic index (MI) (Mamur et al. 2012).

Micronucleus (MN) test

For the MN assay, human lymphocyte culture was incubated at 37 °C for 72 h. At 44 h after the initiation of the culture, cytochalasin B (Cyt-B) was added at a

final concentration of 5.2 µg/mL to arrest cytokinesis. The cells were treated with hypotonic solution (0.075 M KCl for 5 min) and fixed in a mixture of methanol: glacial acetic acid (3:1, V/V) supplemented with formaldehyde according to the Palus et al.'s (2003) method with some modifications. The slides were air-dried and stained in 5 % Giemsa for 13 min. Micronuclei were scored in 1,000 binucleated cells per donor (totally 2,000 binucleated cells per concentration). 500 lymphocytes from each donor (totally 1,000 lymphocytes) were scored to evaluate the percentage of cells with 1–4 nuclei. The cytokinesis-block proliferation index (CBPI) was calculated as follows: $[1 \times N_1] + [2 \times N_2] + [3 \times (N_3 + N_4)]/N$, where, N_1 – N_4 represent the number of cells with 1–4 nuclei, respectively, and N is the total number of cells scored (Mamur et al. 2012).

Statistical analysis

For statistical analysis of the results, the z test was applied for the percentage of abnormal cells, CA/cell, RI, MI, the percentage of MN and CBPI and, the t test for SCE analyses. Dose–response relationships were determined from correlation and regression coefficients for the percentage of abnormal cell, CA/cell, SCE and mean MN.

Results

Results obtained in this study were given in Tables 1–3. Table 1 shows anticlastogenic effect of LA on MMC induced CA in human lymphocyte. In lymphocytes treated with MMC alone, statistically significant ($p < 0.001$) increase was observed in the frequency of CA and CA/Cell compared to the negative and solvent controls. MMC induced chromatid and chromosome breaks, acentric fragments, chromatid exchanges, dicentric chromosomes and sister chromatid unions in human lymphocytes. Chromatid breaks and chromatid exchanges such as triradials and quadriradials were the dominant types of aberrations. In lymphocytes treated with MMC + LA, the frequency of aberrations and the number of CA/cell decreased significantly for all treatment types (1 h pre-treatment, simultaneous treatment and 1 h post-treatment) and for all treatment concentrations (0.5, 1.0 and 2.0 µg/mL) compared to MMC alone. However, this decrease was

Table 1 Effect of MMC + LA on the frequency of chromosome aberrations in cultured human lymphocytes

Test substance	Treatment		Aberrations									Abnormal cell ± SE (%)	CA/ Cell ± SE
	Time (hour)	Doses (µg/mL)	ctb	csb	f	scu	dic	cte	p	Total			
											MMC		
Negative control	48	–	–	5	–	–	1	–	–	–	6	3.0 ± 0.01	0.03 ± 0.01
Solvent control	48	–	–	4	–	–	2	1	–	–	7	3.5 ± 0.01	0.04 ± 0.01
Positive control (MMC)	48	0.2	–	54	9	10	15	26	41	–	155	56.5 ± 0.04	0.78 ± 0.03
MMC + LA (1 h pre-treatment)	49	0.2	0.5	32	–	4	7	12	38	–	93	38.0 ± 0.03**	0.47 ± 0.04**
		0.2	1	23	4	9	9	9	23	2	78	32.0 ± 0.03**	0.39 ± 0.03**
		0.2	2	43	1	2	2	9	41	–	98	35.0 ± 0.04**	0.49 ± 0.04**
MMC + LA (simultaneous treatment)	48	0.2	0.5	32	2	9	9	18	20	–	90	37.0 ± 0.03**	0.45 ± 0.04**
		0.2	1	31	3	9	7	15	31	–	96	37.5 ± 0.03**	0.48 ± 0.04**
		0.2	2	31	3	2	4	11	41	–	92	34.5 ± 0.03**	0.46 ± 0.04**
MMC + LA (1 h post-treatment)	47	0.2	0.5	37	4	11	7	12	30	1	102	38.5 ± 0.03**	0.51 ± 0.04**
		0.2	1	37	2	4	6	17	39	–	105	40.5 ± 0.04*	0.53 ± 0.04**
		0.2	2	29	4	2	6	12	51	–	105	43.0 ± 0.04*	0.53 ± 0.04**

* Significantly different at $p < 0.01$ when compared with positive control (z test)

** Significantly different at $p < 0.001$ when compared with positive control (z test)

MMC Mitomycin-C, LA Lipoic acid, ctb chromatid break, csb chromosome break, f fragment, scu sister chromatid union, dic dicentric, cte chromatid exchange, p polyploidy, CA/cell Chromosome aberrations/cell, SE Standard Error

not dose-dependent. One h pre-treatment and simultaneous treatment of LA caused the more effective reduction (Table 1).

Table 2 shows the effect of LA on the frequency of SCE per cell, replication index and mitotic index in MMC treated human lymphocytes in culture. In cells treated with MMC alone, significant increase in SCE per cell and a significant decrease in mitotic index was observed compared to negative and solvent controls. Replication index decreased with MMC treatment but this decrease was not significant. On the other hand, all treatment types (1 h pre-treatment, simultaneous treatment and 1 h post-treatment) and treatment concentrations (0.5, 1.0 and 2.0 $\mu\text{g/mL}$) of LA together with MMC significantly decreased the number of SCEs/cell compared to MMC alone. This reduction was most effective in 1 h pre-treatment group and at 1 and 2 $\mu\text{g/mL}$ concentrations. In all MMC + LA supplemented cultures, replication index decreased compared to MMC, negative and solvent controls. However RI revealed no significant alternation compared to MMC alone. For the mitotic index, a significant and dose dependent decrease ($r = 0.998$, $r = 1.00$, $r = 0.975$, for pre-, simultaneous and post-treatments, respectively) was observed in all the

cultures treated with MMC + LA compared to MMC alone.

Table 3 summarizes the effect of MMC alone and MMC + LA on the frequency of MN and CBPI in cultured human lymphocytes. MMC significantly increased the frequency of micronuclei compared to negative and solvent controls. On the contrary, MMC + LA treatments significantly reduced the frequency of micronuclei in the three concentrations (0.5, 1.0 and 2.0 $\mu\text{g/mL}$) and in the three treatment groups (1 h pre-treatment, simultaneous treatment and 1 h post-treatment). In the three treatment groups, the CBPI value decreased dose dependently but it was significant only for the 1 and 2 $\mu\text{g/mL}$ concentrations.

Discussion

The frequency of CA in peripheral blood lymphocytes has been applied for decades as a biomarker of the early effects of genotoxic carcinogens, in occupational and environmental settings (Bonassi et al. 1995; Albertini et al. 2000; Bonassi et al. 2005; Ünal et al. 2011; Mamur et al. 2012). CAs in lymphocytes are thought to represent a surrogate endpoint for more

Table 2 Effect of MMC + LA on the frequency of sister chromatid exchange, replication index and mitotic index in cultured human lymphocytes

Test substance	Treatment		Min–max SCE	SCE's/ cell ± SE	M1	M2	M3	RI ± SE	MI ± SE	
	Time (hour)	Dose (µg/ml)								
		MMC								LA
Negative control	48	–	–	2–8	4.46 ± 0.22	5	82	113	2.54 ± 0.039	9.60 ± 0.20
Solvent control	48	–	–	1–9	4.60 ± 0.29	4	87	109	2.53 ± 0.038	9.05 ± 0.19
Positive Control (MMC)	48	0.2	–	38–78	51.16 ± 1.32	82	105	13	1.66 ± 0.039	8.05 ± 0.18
MMC + LA (1 h pre-treatment)	49	0.2	0.5	25–65	37.26 ± 1.05 ^a	99	93	8	1.55 ± 0.040	6.20 ± 0.13**
		0.2	1	19–48	31.36 ± 0.98 ^a	92	102	6	1.57 ± 0.039	5.65 ± 0.12**
		0.2	2	21–45	30.28 ± 0.87 ^a	106	89	5	1.50 ± 0.039	4.80 ± 0.10**
MMC + LA (simultaneous treatment)	48	0.2	0.5	24–53	37.78 ± 0.86 ^a	90	103	7	1.59 ± 0.039	7.40 ± 0.15**
		0.2	1	23–58	35.30 ± 1.32 ^a	105	87	8	1.52 ± 0.040	6.90 ± 0.14**
		0.2	2	17–72	33.08 ± 1.89 ^a	103	90	7	1.52 ± 0.040	5.85 ± 0.12**
MMC + LA (1 h post-treatment)	47	0.2	0.5	23–62	37.80 ± 1.23 ^a	85	103	12	1.64 ± 0.040	7.55 ± 0.16**
		0.2	1	18–74	41.70 ± 1.78 ^a	114	76	10	1.48 ± 0.040	7.10 ± 0.15**
		0.2	2	19–62	35.70 ± 1.66 ^a	116	75	9	1.47 ± 0.040	6.70 ± 0.14**

^a Significantly different at $p < 0.05$ when compared with positive control (t test)

** Significantly different at $p < 0.001$ when compared with positive control (z -test)

MMC Mitomycin-C, LA Lipoic acid, SCE's/cell Sister chromatid exchange, M_1 mitosis 1, M_2 mitosis 2, M_3 mitosis 3, RI replication index, MI mitotic index, SE standard error

Table 3 Effect of MMC + LA on the frequency of micronucleus and cytokinesis block proliferation index in cultured human lymphocytes

Test substance	Treatment			BN cells scored	Distrubition of BN cells according to the no of MN			MN (%) ± SE	Cytokinesis-block proliferation index (CBPI) ± SE
	Time (hour)	Dose (µg/ml)							
		MMC	LA		(1)	(2)	(3)		
Negative control	48	–	–	2,000	4	–	–	0.20 ± 0.009	1.95 ± 0.043
Solvent control	48	–	–	2,000	9	1	–	0.55 ± 0.011	1.93 ± 0.042
Positive control (MMC)	48	0.2	–	2,000	153	9	–	8.55 ± 0.18	1.86 ± 0.40
MMC + LA (1 h pre-treatment)	49	0.2	0.5	2,000	98	2	–	5.10 ± 0.10 **	1.87 ± 0.040
		0.2	1	2,000	100	8	–	5.80 ± 0.12 **	1.68 ± 0.034**
		0.2	2	2,000	100	15	–	6.75 ± 0.14 **	1.52 ± 0.028**
MMC + LA (simultaneous treatment)	48	0.2	0.5	2,000	130	8	–	7.30 ± 0.15 **	1.90 ± 0.041
		0.2	0.5	2,000	123	8	–	6.95 ± 0.14 **	1.74 ± 0.036*
		0.2	0.5	2,000	118	12	4	7.70 ± 0.16	1.58 ± 0.030**
MMC + LA (1 h post-treatment)	47	0.2	1	2,000	122	8	–	6.90 ± 0.14 **	1.78 ± 0.037
		0.2	2	2,000	126	14	–	7.70 ± 0.16 **	1.68 ± 0.034**
		0.2	1	2,000	133	9	–	7.55 ± 0.16 **	1.58 ± 0.030**

* Significantly different at $p < 0.01$ when compared with positive control (z test)

** Significantly different at $p < 0.001$ when compared with positive control (z test)

MMC Mitomycin-C, LA Lipoic acid, BN Binucleat, MN micronucleus, SE standard error

specific chromosome alterations in target tissues of carcinogenesis. Assuming that the mechanisms of chromosome damage formation are similar in different tissues, the level of damage in lymphocytes can be expected to reflect the level of damage in cancer-prone tissues and to indicate cancer risk (Bonassi et al. 1995; Albertini et al. 2000; Obe et al. 2002; Bonassi et al. 2005; Norppa et al. 2006). Chromosomal aberration frequency in peripheral lymphocytes of healthy individuals has been found to be predictive of future cancer incidence in several epidemiologic studies (Bonassi et al. 1995; Hagmar et al. 1998; Rossner et al. 2005; Boffetta et al. 2007). Bonassi et al. (2008) reported in their cohort study of 22,358 subjects from 11 countries that chromosomal aberration frequency measured in peripheral blood lymphocytes of healthy subjects is associated with cancer risk. A stronger association was initially described with lung cancer and hematological malignancies (Bonassi et al. 1995), whereas more recently, based on larger numbers, both the Czech cohort (Rossner et al. 2005) and the Central and Eastern European country cohort (Boffetta et al. 2007) reported an increased risk of stomach cancer. Similar result was reported by Bonassi et al. (2008). The micronucleus technique has also been proposed as a useful tool for measuring genotoxicity in *in vitro* cultures. The formation of micronuclei in dividing cells is the result of chromosome breakage due to unrepaired or misrepaired DNA lesions, or chromosome missegregation due to mitotic dysfunction (Fenech and Morley 1985; Fenech 2006; 2007; Mamur et al. 2012). These events may be induced by oxidative stress, exposure to clastogens or aneugens, genetic defects in cell cycle checkpoint and/or DNA repair genes, as well as deficiencies in nutrients required as co-factors in DNA metabolism and chromosome segregation machinery (Bonassi et al. 2007, 2008). Formation of MN is associated with the chromosome instability often seen in cancer (Bonassi et al. 2005; Fenech 2006, 2007; Iarmarcovai et al. 2008; Burgaz et al. 2011). The hypothesis of an association between MN frequency and cancer development is supported by a number of observations. The most substantiated ones include: (1) the high frequency of this biomarker in untreated cancer patients and in subjects affected by cancer-prone congenital diseases, (2) the correlation existing between genotoxic MN-inducing agents and carcinogenicity, and (3) the inverse correlation between MN frequency and

the blood concentration and/or dietary intake of certain micronutrients associated with reduced cancer risk (Iarmarcovai et al. 2008; Burgaz et al. 2011; El-Zein et al. 2011). SCE interchanges of DNA replication products between sister chromatids at apparently homologous loci, have long been applied in surveillance of human genotoxic exposure and early effects of genotoxic carcinogens (Albertini et al. 2000; Norppa et al. 2006). SCEs are known to reflect the repair of DNA lesions by homologous recombination (Norppa et al. 2006). As both genotoxic exposure and DNA repair capacity are expected to differ among individuals, the association of SCE level with cancer risk may be difficult to predict. However, SCEs remain a convenient tool in experimental studies and are used as a biomarker of human genotoxic effects (Norppa et al. 2006). The genotoxic end-points mentioned above are well known markers of genotoxicity and any reduction in their frequency gives an indication of the antigenotoxicity of a particular compound (Albertini et al. 2000; Bilska and Wlodek 2005; De Flora and Ferguson 2005; Begum et al. 2012).

Genotoxic effects of ionizing radiation and anti-cancer drugs in non-tumor cells are of special significance due to the possibility that they may interact with genetic material of normal cells and induce secondary tumors in cancer patients (Siddique et al. 2008; Begum et al. 2012; dos Santos et al. 2012). These therapies have harmful effects on health-care persons as well (Aydemir et al. 2005; McDiarmid et al. 2010). On the other hand, in recent years, some natural products are used in the form of dietary supplements for their possible health promoting effects such as their potential in preventing cancer, atherosclerosis, heart disease, etc. (D'Incalci et al. 2005; Scarpato et al. 2008; Bengmark et al. 2009; Del Rio et al. 2010; Begum et al. 2012). It is reported that uptake of plant products may modulate the genotoxicity of anti-cancer treatments and may reduce the chances of developing secondary tumors in cancer patients. The food phytochemicals are naturally occurring antioxidants with known chemo-preventive effects against oxidative stress and DNA damage in a wide range of *in vitro* and *in vivo* assay systems (D'Incalci et al. 2005; Abraham et al. 2012). Currently it has been reported that various medical plants and phenolic compounds ameliorate cytogenetic alterations in cultured human blood lymphocytes (Ananthi et al. 2010; Begum et al. 2012; Bhourri et al. 2012; Montoro et al. 2012; Turkez et al.

2012). A recent work carried out by Abraham et al. (2012) demonstrated significant protective effects of the food phytochemicals pelargonidin, chlorogenic acid, resveratrol and epigallocatechin gallate against the *in vitro* genotoxicity of mitomycin C, diepoxybutane and patulin, when these phytochemicals are tested either as single agents or in combination. Natural products are rich in antioxidants which exhibit their effects at different levels. These include ability to scavenge primary and secondary radicals, to inhibit free-radical-induced membrane damage and potential to bind iron that can prevent radical formation (Maurya and Devasagayam 2010; Tian et al. 2012). A large number of phytochemicals, which possess antioxidant and free-radical scavenging properties, are known to modulate important cellular signaling pathways associated with carcinogenesis (Surh 2003; Lee et al. 2011). MMC, an antibiotic, has a range of genotoxic effects including selective inhibition of DNA synthesis, mutagenesis, chromosome breakage and sister chromatid exchange (Tomasz 1995). MMC is a direct acting clastogen (Grisolia 2002; Scarpato et al. 2008), requiring only intra-cellular reductive activation to initiate its potent DNA cross-linking action. MMC cross-links the complementary strands of the DNA double helix. Crosslinking agents, typically bifunctional alkylation agents like MMC is used in cancer chemotherapy (Tomasz 1995; Bounias et al. 1997; Gentile et al. 1998; Grisolia 2002; Prahalathan et al. 2006).

The objective of this study was to investigate the protective effect of the antioxidant LA against MMC-induced genotoxicity in human peripheral lymphocytes by using chromosomal aberrations, SCE and micronucleus tests. To demonstrate anticlastogenic properties of LA, untreated, solvent and positive controls were also included in the experiments. In all the experiments, MMC treatment significantly increased the number of CA per cell, the percentage of abnormal cells and micronuclei and the number of SCE per cell compared with negative and solvent controls. In the presence of LA together with MMC, the frequency of abnormal cell and CA/cell decreased significantly in the three different treatment groups for all concentrations compared with the group treated with MMC. However, the number of these damages was not reduced to the level of control. 1 h pre-treatment of LA was most effective in protecting against MMC induced aberrations. Simultaneous and

1 h post-treatment of LA also showed statistically significant reduction in the frequency of chromosome aberrations and CA/cell in comparison with MMC treated cultures. LA treatments significantly decreased SCE/cell and the frequency of micronuclei induced by MMC alone. Again, 1 h pre-treatment of LA was most effective to protect against MMC induced damages. Although the test concentrations did not reduce damages to the control level, LA showed protective effect against MMC induced genotoxicity in human peripheral lymphocytes. LA successfully reduced the clastogenic effects of some other antitumor drugs such as cyclophosphamide and adriamycin in *in vivo* treatments (Prahalathan et al. 2006; Selvakumar et al. 2006a, b; Novonty et al. 2008). In experimental cancer therapy and cancer chemotherapy in humans, LA can decrease the toxicity of anticancer drugs which are known to cause high rates of free radical formation. Scavenging of reactive molecules represents one of the approaches in antimutagenesis and anticarcinogenesis (Selvakumar et al. 2006b; Shay et al. 2009). LA and its reduced form DHLA have the ability to scavenge the singlet oxygen, hydrogen peroxide, hydroxyl radicals, superoxide radicals and also to chelate the ferrous ion involved in the production of hydroxyl radical (Sewierenga et al. 1991; Ho et al. 2007; Shay et al. 2009; Lee and Na 2010). Lipoic acid also regenerates other antioxidants like glutathione, vitamin C and vitamin E, which reduces oxidative stress *in vivo* (Packer et al. 1997; Selvakumar et al. 2006b; Lee and Na 2010; Tian et al. 2012). The mechanism of antioxidant effects of LA in rat brain is related to activation of the nonenzymatic and enzymatic antioxidant defense systems (Pronko et al. 2006). The possibilities of protection by LA may also include hydrogen ion donation for DNA repair and inhibition of alkylating agents when the thiol component attracts the positively charged carbonium ions (Selvakumar et al. 2006a, b). LA has the ability to correct deficient thiol status by increasing the uptake of cysteine by cells, leading to increased glutathione synthesis (Han et al. 1997; Suh et al. 2004a, b; Lee and Na 2010; Tian et al. 2012). The beneficial action of LA may result in its ability to reduce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase/endothelial cell-mediated ROS generation, restore GSH/GSSG contents and enhance the mitochondrial expression of key antioxidant enzymes, including glutathione reductase (Bitar et al. 2010). In experiments *in vivo* and

in vitro LA prevented ethanol-induced neurotoxicity and intracellular protein oxidation (Pierce et al. 2006). LA induces cell cycle arrest and apoptosis in cancer cells such as leukemia and colon cancer transformed cells but not in normal cells (Pierce et al. 2000; Wenzel et al. 2005). It has recently been shown that the application of LA to a human breast cancer cell line inhibits cancer metastasis through a decrease in the activity and mRNA expression levels of MMP-2 and MMP-9. These 2 metalloproteinases (MMPs) were shown to be expressed in tumor cells. Therefore, inhibition of MMP activity by dietary factors holds great promise for the prevention or inhibition of metastasis (Lee and Na 2010). Lipopolysaccharide induced nitric oxide and prostaglandin E2, two important mediators associated with inflammation, can be inhibited by DHLA and LA indicating the possibility that DHLA/LA could have beneficial effects on preventing several other diseases mediated by NO and PGE2 overproduction (Ho et al. 2007). Xiao et al. (2012) investigated the protective effects of LA on the hydrogen peroxide-induced oxidative stress and impaired calcification in MC3T3-E1 osteoblast-like cells. The presence of LA attenuated oxidative stress and significantly enhanced calcification capacity. The quantitative real-time PCR results showed that LA upregulated expression of genes engaged in antioxidant enzymes and mineralization, including catalase, glutathione peroxidase 3, and osteocalcin. Additionally, LA suppressed the over-expression of genes involved in p53 apoptotic pathway, including p53 and caspase 3. These results indicate that LA protects MC3T3-E1 cells against the H₂O₂-induced oxidative damage to mineralization (Goraca and Aslanowicz-Antkowiak 2009a; Goraca et al. 2009b; Xiao et al. 2012).

Beside its antioxidant role, in vitro and in vivo studies suggest that LA and its reduced form DHLA also act as a pro-oxidant (Moini et al. 2002; Çakatay 2006; Goraca and Aslanowicz-Antkowiak 2009a; Goraca et al. 2009b). DHLA accelerates iron dependent hydroxyl radical generation and lipid peroxidation in liposomes, probably by reducing Fe³⁺ to Fe²⁺. Increased Fe³⁺ reduction can result in prooxidant activity. Displacement of balance between pro- and antioxidant can account for mechanisms involved in etiopathogenesis and/or progression of many pathological states, like tumors, AIDS, neurodegenerative diseases, diabetes etc. (Bilska and Wlodek 2005; Tian

et al. 2012). The prooxidant activity of LA may depend on the type of oxidant stress and the physiological circumstances of the cell. Under different conditions, LA can act as a pro-oxidant (Ho et al. 2007; Lee et al. 2011).

In this study, the possible preventive efficacy of LA against the effect of MMC on the mitotic activity was also investigated. LA treatments induced significant dose dependent reduction ($r = 0.998$, $r = 1.00$, $r = 0.975$ for pre-, simultaneous and post-treatments, respectively) in mitotic activity in all cultures. Though the reduction of mitotic index is thought as the indicator of cytotoxicity, our observations suggest an inhibitory role of the antioxidant LA on the proliferation of mitogen-stimulated peripheral blood lymphocytes. Pack et al. (2001) reported that, the activation and proliferation of T lymphocytes is intimately linked to the intracellular redox state of the cell. A number of studies have shown that various antioxidant agents, including free radical scavengers, iron chelators, and electron acceptors, can inhibit lymphocyte proliferation. The inhibition of mitotic activity may also depend on the hydroxyl radical scavenger property of LA (Pack et al. 2001). Agents such as benzoate, thiourea, dimethylurea, tetramethylurea, L-tryptophan, mannitol, and several other chemicals that are known to be scavengers of hydroxyl radicals inhibit lymphocyte mitogenesis (Sen et al. 1999). Since OH[•] radicals are obligatory mediators for the transduction of the mitogenic signal, and these agents share the property of scavenging OH[•], they also inhibit lymphocyte mitogenesis (Novogrodsky et al. 1982).

In summary, this study clearly demonstrates anti-clastogenic and antimutagenic effects of LA against MMC induced genetic damage in human lymphocytes in culture. LA seems to be an ideal antioxidant because of several properties, including (a) the ability to decrease the formation of reactive oxygen species; (b) the ability to scavenge reactive oxygen species, including OH[•], O₂^{•-}, alkoxy radicals, and peroxide radicals; (c) the ability to regenerate other antioxidants such as vitamins E and C and GSH from their radical or inactive forms; (d) metal chelating activity; and (e) enhancing the DNA repair system or DNA synthesis or some other ways (Biewenga et al. 1997; De Flora and Ferguson 2005; Cremer et al. 2006; Fu et al. 2008; Davis et al. 2009; Lee and Na 2010; Tian et al. 2012). It is known that mutations in somatic cells play a key role in some chronic diseases, initiation of

cancer and other strategies of the carcinogenesis. It is also known that anticlastogenesis is an essential part of antimutagenesis and anticarcinogenesis. Furthermore, certain prevention strategies applicable to cancer prevention are also beneficial to prevent other chronic diseases (Gebhart 1992; Ho et al. 2007; Lee and Na 2010; Tian et al. 2012). It is concluded that anticlastogenic and antimutagenic agents like LA may play a vital role not only preventing the development of cancer and other chronic diseases but also in protecting people exposed to irradiation and chemotherapeutic agents by reducing the chances of developing secondary tumors. On the other hand, LA and DHLA may also act as a pro-oxidant, probably by reducing Fe^{+3} to Fe^{+2} , which should be carefully taken into account in risk–benefit analyses (Lu and Kang 2012).

In conclusion, it is demonstrated in this *in vitro* study that LA has a protective effect against MMC induced genetic damage. These results are encouraging and LA can be a possible chemopreventive agent in tumorigenesis in both cancer patients and in health care persons handling anti-cancer drugs.

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