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Inhibition of Induced Micronuclei Formation in Human Lymphocytes by Ginger

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Abstract: Spices and condiments consumed in Indian diets are one of the sources of phytochemicals and may act as antigenotoxicants. This study was taken up to evaluate the antigenotoxic potential of ginger in human peripheral blood lymphocytes. Peripheral blood samples from apparently normal 18 males (9 male smokers and 9 male non smokers) and 9 females were obtained. 0.5 mL of whole blood in a total volume of 5 mL medium was cultured in the presence of 0.16 mM Trans Stilbene Oxide (TSO) for the induction of micronuclei. Aqueous ginger extract was added at concentrations namely 0.1, 0.2 and 0.4 mg to test for antigenotoxicity. After 72 h, the cultures were processed and the cell suspension was fixed on slides and stained with 2% giemsa. The micronuclei were scored in 1000 binucleated cells and the data was analysed by analysis of variance (ANOVA). It was found that at basal condition without ginger and TSO the Micronuclei frequency was higher in smokers compared to non-smokers. *In vitro* treatment of peripheral blood lymphocytes with TSO induced significant number of micronuclei in all samples. Simultaneous treatment with ginger extract at all the concentrations significantly $p < 0.001$ inhibited formation of micronuclei. A dose response relationship was observed. The extent to which ginger extract reduced the micronuclei formation induced by TSO exposure was almost normal or less than the control values. However, there were no changes in the vit. A and vit. E levels in all the groups. Results of the study indicate that ginger may protect against chromosomal damage induced by genotoxicant, as evidenced by this *in vitro* experimentation.

Key words: Trans stilbene oxide, lymphocyte micronucleus test, peripheral blood lymphocytes, cytokinesis, ginger, chemopreventer

INTRODUCTION

Large number of studies has revealed that a regular consumption of fruits and vegetables provide good protection against cancer at many sites and some of the foods and herbs contain a host of cancer-protective phytochemicals (Hung *et al.*, 2006). These beneficial compounds alter metabolic pathways and hormonal actions that are associated with the development of cancer, stimulate the immune system and have antioxidant activity (Craig, 1999).

Mutagenicity, clastogenicity, cytotoxicity and carcinogenicity are inhibited by antioxidant compounds found in abundance in plants (Hochstein and Atallah, 1988). Most chemopreventive compounds and their analogs and derivatives are initially of plant origin and inhibit spontaneous and chemical mutagenesis in a variety of *in vitro* and *in vivo* test systems (Xifeng *et al.*, 2007). The screening of crude plant extracts for antimutagenic and anticarcinogenic activity has led to the identification of a variety of compounds (Wall *et al.*, 1990; Renner, 1990).

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In recent times, spices are being discovered to have antimutagenic and anticarcinogenic potentials and there has been substantial evidence of data, supporting that dietary factors have a profound effect on prevention and etiology of human cancer (Weisburger, 2000). Some pungent constituents of ginger have potent antioxidant and anti-inflammatory effects and some of them exhibit antitumour promotional activity in experimental carcinogenesis (Kirana *et al.*, 2003). From a large number of Zingiberaceae species that have been used for culinary and/or medicinal purposes, only few members have studied for their potential anticancer activity. Murakami *et al.* (2002) reported that Zerumbone, which was isolated from *Zingiber aromaticum* and *Zingiber zerumbet* had an antiproliferative effect on various human colonic adenocarcinoma cell lines. However the molecular mechanisms by which they exert their antitumorogenic effects are unknown (Bode *et al.*, 2001).

There are various ways of detecting the antigenotoxic potential of these substances from short-term genotoxicity assays *in vitro* to bioassays in humans. These techniques focus on the use of biomarkers to study at biochemical, physiological, enzymatic or cellular level, the changes recurring between external exposure and eventual effect. It is essential to evaluate promising nutrients and non-nutrients in foods as chemopreventive agents in persons at increased risk of cancer. Development of reliable intermediate biomarkers is valuable for clinical, chemoprevention intervention trials (Bayoumy *et al.*, 1997; Kirsch-Volders and Fenech, 2001). In an *in vitro* experiment on ginger it was shown that ginger has potential to inhibit carcinogen induced cell damage by benzo (a) pyrene as measured by comet assay (Nirmala *et al.*, 2007a).

In the present study, the *in vitro* micronucleus (MN) formation was investigated in cytokinesis-blocked human peripheral blood lymphocytes following *in vitro* exposure to Trans Stilbene Oxide (TSO) (Miller *et al.*, 1995; Van Hummelin and Kirsch-Volders, 1992) in the presence of ginger extract at various levels. The micronucleus assay is a reliable biomarker of genotoxicity and can also be employed for identifying compounds that might either inhibit clastogenesis or genotoxicity (Stankova and Novak, 2003).

Using the Cytokinesis-Block Micronucleus assay (CBMN) (Fenech *et al.*, 2003), chromosomal damage in cells can be selectively assessed in cells that complete one nuclear division following *in vitro* treatment with mutagen.

MATERIALS AND METHODS

Preparation of Ginger Extract

Ten grams of fresh ginger was taken and made into paste and 25 mL of water was added to it and left overnight in shaker at room temperature. The next day it was filtered and the supernatant was collected and lyophilized. This extract was used in the assay, by diluting it to 10 mg mL⁻¹ with water and from this solution 10, 20 and 40 µL was taken for the assay, which corresponds to 0.1, 0.2 and 0.4 mg of ginger extract, respectively.

Study Design

The study was conducted at National Institute of Nutrition, India, by following the Institutes Ethical clearance committee. Peripheral blood samples from eighteen males (9 male smokers and 9 male non-smokers) and 9 females were obtained. The human volunteers were apparently normal and the age groups ranged from 35-55 years. The samples obtained from these subjects were used to induce MN with TSO. Blood samples were also collected from a group of 6 male non-smokers, 6 male smokers and 6 females to evaluate the effect of different concentrations of ginger treatment in the absence of trans stilbene oxide on the incidence of micronuclei. Cell viability was tested by trypan blue exclusion method.

Culture Medium

To a flask containing 500 mL of GDW, 25 mM HEPES (sigma) and 0.6 g of sodium bicarbonate was added and mixed well. 4.9 g of F10 mix (Himedia) was added to the mixture and was autoclaved. The medium was supplemented with 150 mL of heat inactivated foetal calf serum (Sigma) (30 min at 56°C), 400 IU of heparin, 100 IU of penicillin and streptomycin and phytohaemagglutinin-C (Boehringer-Mannheim Co.) (45 mg dissolved in 5 mL of medium). This complete growth medium was dispensed in screw-capped tubes (4.5 mL tube⁻¹) and was kept at -20°C until use.

Cytochalasin B

Three milligram was dissolved in 10 mL of 1:1 dimethyl sulfoxide and water and was refrigerated at -20°C. It was melted in water bath at 37°C before adding to the cultures. 0.1 mL was added to 5 mL of the culture medium (30 µg tube⁻¹).

Hypotonic Solution

Potassium chloride (5.6 g L⁻¹) was prepared fresh on the same day of assay and stored at 4°C in refrigerator.

Fixative

Mixture of methanol/acetic acid (3:1) was prepared fresh just before use.

Culture and Treatment

Point five millilitre of heparinised whole blood was added to 4.5 mL of culture medium in screw capped vials. A concentration of 0.16 mM of TSO in a volume of 40 µL was added to cultures. Ginger extract at different levels (0.1, 0.2 and 0.4 mg) were added to the cultures and incubated at 37°C for 72 h. Cytochalasin B was added at the 44th h after setting the cultures.

Fixation

The cultures were removed from incubation after 72 h, transferred into centrifuge tubes and centrifuged at 800 rpm for 8 min. The supernatant was discarded and the pellet was suspended in 5 mL of ice-cold 75 mM KCl (hypotonic solution), centrifuged at 800 rpm for 8 min. The supernatant was removed and the pellet was resuspended in 5 mL of fixative. While vortexing slowly, 2-3 drops of formalin was added and centrifuged at 800 rpm for 8 min. The cells were further washed with fixative two times and after centrifugation supernatant was discarded and the pellet was suspended in 0.5 mL of fixative.

Slide Preparation

The cell suspension was dropped on ice cold slides using drawn out pasteur pipette. The drops after expanding and when the Newton rings appeared the cells were exposed to infrared lamp for a minute.

Slide Staining and Scoring

The slides were stained in 2% aqueous giemsa solution, rinsed for few minutes in distilled water and air-dried. For each treatment micronuclei were scored in 1000 binucleated cells. The data was analysed by ANOVA using SPSS 10.0 window version.

Micronutrients

Retinol (vit. A), Retinyl acetate (internal standard) and α -tocopherol (vit. E), were purchased from Sigma Chemical Co. HPLC grade methanol, water and n-Hexane, were obtained from Qualigens and Spectrochem Ltd., India.

Analysis of Vit. A and Vit. E

Plasma from whole blood was analysed simultaneously for vit. A and vit. E by HPLC following the method of Bieri *et al.* (1979).

Statistical Analysis

The data was analyzed by the analysis of variance (ANOVA). Testing of mean values in different groups was done by Duncan's multiple range test. SPSS 10 Window version was used for the statistical analysis (Middle Brooks, 1977).

RESULTS

In order to understand the role of ginger in human peripheral blood lymphocytes without TSO exposure, a study was undertaken. Six subjects were taken from each group (non-smokers, smokers and females) and mononuclear cell cultures from each sample were incubated with different concentrations (0.1, 0.2 and 0.4 mg of ginger extract) (Table 1). It was found that ginger did not exert any cytotoxic effect and the MN yield reduced with the increasing concentrations of ginger compared to the basal values. It was found that in the absence of ginger the MN yield was 20 ± 1.5 in non-smokers; 27 ± 3.7 in smokers; 20 ± 1.7 in females. In the presence of ginger at various concentrations without TSO exposure the MN yield was reduced with increasing concentrations of ginger and at the higher concentrations of ginger (0.4 mg), the MN yield was 6.7 ± 0.9 in non-smokers; 10 ± 0.9 in smokers; 8 ± 0.9 in females (Table 2).

To assess the effect of ginger in modifying the TSO-induced micronuclei (MN) yield in human peripheral blood lymphocytes (PBL), CBMN assay was conducted in blood samples obtained from normal healthy volunteers ($n = 27$ of which 9 in each were smokers, non-smokers and females). Mononuclear cell cultures from each sample were incubated with different concentrations (0.1, 0.2 and 0.4 mg) of aqueous ginger extract. It was found that at basal condition without ginger and TSO the MN yield was 18 ± 0.9 in male non-smokers; 32 ± 3.3 in male smokers and 24 ± 3.2 in females.

With TSO exposure MN yield sharply increased to 33 ± 3.6 in non-smokers per 1000 binucleated cells, 51.1 ± 4.2 in male smokers and 41 ± 5.5 in females. However treatment with ginger at various concentrations resulted in a significant linear decline of MN yield with the increasing concentration of ginger (Table 3). In the presence of ginger at various concentrations with TSO exposure, the MN yield was reduced with increasing concentration of ginger and at the higher concentration (0.4 mg) the MN yield was 10.3 ± 1.00 in non-smokers; 12.0 ± 1.40 in smokers and 12.8 ± 2.7 in females. Compared to TSO, the extent to which ginger extract reduced the micronuclei yield induced by TSO exposure was almost normal or less than the control values. The results indicated that ginger extract did not exert cytogenetic effect on human PBL at concentrations upto 0.4 mg as evaluated by the CBMN assay and the protection of ginger against TSO induced MN in human PBL is concentration dependence. There

Table 1: Vit. A and vit. E levels in male smokers, male non-smokers and females

Micronutrients	Smokers	Non-smokers	Females
Vitamin A ($\mu\text{g dL}^{-1}$)	69.70 ± 27.19	44.80 ± 10.86	41.50 ± 13.49
Vitamin E (mg dL^{-1})	1.10 ± 0.617	0.86 ± 0.277	0.97 ± 0.35

Values are Mean \pm SD of 9 subjects per group

Table 2: Inhibition of micronuclei in lymphocytes by Ginger Extract (GE) No. of micronuclei per 1000 BN cells

Treatments	Male non-smokers	Male smokers	Females
Control	20.0 ± 1.50^a	27.3 ± 3.70^a	20.3 ± 1.70^a
0.1 mg GE	13.7 ± 0.95^a	23.1 ± 2.90^a	13.0 ± 1.10^a
0.2 mg GE	10.7 ± 0.99^{ab}	15.0 ± 1.40^{ab}	11.0 ± 1.20^{ab}
0.4 mg GE	6.7 ± 0.98^{bc}	10.3 ± 0.95^b	7.6 ± 0.90^{bc}

Values are Mean \pm SE of 6 subjects per group. Different superscripts are significant at $p < 0.05$ by Duncan's multiple range test

Table 3: Inhibition of TSO induced micronuclei in lymphocytes by Ginger Extract (GE)

Treatments	No. of micronuclei per 1000 BN cells		
	Male non-smokers	Male smokers	Females
Control	18.2±0.89	32.6±3.30 ^a	24.2±3.21 ^a
TSO	32.6±3.57	51.1±4.24 ^b	40.8±5.48 ^b
TSO + 0.1 mg GE	18.8±1.62	31.1±1.92 ^a	32.4±5.22 ^a
TSO + 0.2 mg GE	14.4±1.29	17.5±1.40 ^c	18.4±4.02 ^{ab}
TSO + 0.4 mg GE	10.3±1.00	12.0±1.35 ^d	12.8±2.72 ^{ac}

Values are Mean±SE of 9 subjects per group. TSO: Trans stilbene oxide. Different superscripts are significant at p<0.05 by Duncan's multiple range test

were no significant differences in the levels of micronutrients Vitamin A and Vitamin E in all the three groups (Table 1). Therefore our findings indicated that ginger might have therapeutic value as a possible chemopreventer by virtue of its anticarcinogenic property.

DISCUSSION

Environmental carcinogens and mutagens induce DNA damage and chromosomal damage. Assessment of genotoxicity can be performed at different steps of the interaction as well as the effects of the mutagen on DNA. The direct DNA breaking capacity can be estimated either by alkaline elution or alkaline gel electrophoresis.

In vivo studies in rats fed with ginger also showed that it has strong antimutagenic and antioxidant potential (Nirmala *et al.*, 2007b, c). These techniques can be also applied to test the antigenotoxic potentials of potential chemopreventers. Depending on the stage of the cell cycle, repair capacity, genetic background of the cells and the nature of mutagen used, only some amount of induced DNA damage will lead to fixed mutations eventually. These genomic mutations can be quantified by determining micronuclei. Reduction in DNA damage/mutation by adopting intakes of diet rich in chemopreventers is likely to reduce risk of developing mutation based diseases.

The cytokinesis-block micronucleus assay in human lymphocytes is a sensitive and valuable technique to detect chromosome mutations and loss, which are important events in the development of cancer. Therefore, experiments were performed to evaluate the antigenotoxic potential of ginger on trans stilbene oxide induced micronuclei in human lymphocytes. Trans stilbene oxide is an epoxide mutagen similar to benzo (a) pyrene 7, 8-diol 9, 10 epoxide (BPDE) which can bind to cellular macromolecules and form adducts (Autrup *et al.*, 2006). TSO has been shown to induce sister chromatid exchanges (Wiencke *et al.*, 1990) in peripheral blood lymphocytes in a study involving 45 normal adult volunteers (19 males; 26 females).

In this study also an increased (p<0.001) incidence of micronucleus was observed following incubation of blood cells with TSO. Ginger extract tested at various concentrations without TSO exposure did not show any adverse effects. On the contrary in the presence of ginger and TSO exposure, reduction in the level of spontaneously occurring micronuclei was observed. A decrease was noted with increasing levels of ginger extracts present in incubation medium.

Mayer *et al.* (2000) have demonstrated positive correlation between lipid peroxidation and increased MN formation in lymphocytes. Oxidative stress due to free radical production and subsequent breakdown of antioxidant defense is one of the positive factors to induce chromosomal breakage and MN formation. The enhanced activity of GST and QR in target tissues like liver, lungs and kidney in ginger treated rats suggest that antigenotoxic effect could be through detoxification pathway (Nirmala *et al.*, 2008).

An elevated basal frequency of MN was observed in male smokers (32.5±3.30) as compared to non-smoking males (18.2±0.09) and females (24.2±3.20). It is known that frequency of micronuclei formation is more in smokers compared to non-smokers (Bonassi *et al.*, 2003). It has been reported

that females have increased MN frequency possibly due to preferential loss of inactive X-chromosome. However other life style factors including dietary habits are important variables influencing the MN incidence. Diet rich in antioxidants including micronutrient vitamins confer protection against cytogenetic damage (Loft and Poulsen, 2000).

Therefore, micronutrient vitamins (Vitamin A and Vitamin E) were estimated in all the samples. The levels of these micronutrients were within normal expected range (Vitamin A normal range: 20-90 $\mu\text{g dL}^{-1}$ and Vitamin E normal range: 0.5-1.5 mg dL^{-1}) (Johanna, 2001). These micronutrients confer protective effect by virtue of their antioxidant property. In a study reported by Fenech and Rinaldi (1994), it was observed that antimutagenic property of micronutrients varied between sexes. High levels of vitamin C and low levels of B₁₂ were associated with elevated MN frequency in young males where as low levels of B₁₂ and folate appeared to interact in elevated MN frequency in young females. The authors suggest that ascorbic acid may destroy B₁₂ *in vitro* (Fenech and Ferguson, 2001). The protective effect of ginger extract against TSO induced cytogenetic damage at various concentrations suggest that non-nutrient component of the diet can prevent genetic damage and have no interactive as is observed with antioxidant micronutrient vitamins. In fact, high levels of antioxidant vitamins may show adverse effect (Fairfield and Fletcher, 2002). Reports on modulatory effects of antioxidants like carotenoids are conflicting. Short intervention trials with carotenoids in human did not show prevention of oxidative damage (Van Poppel *et al.*, 1995). Carotenoids are easily degraded and undergo auto oxidation. The antioxidant effect of β -carotene depends on oxygen pressure as a result of competition between two reactions, one producing a chain terminator peroxy radical and other producing a chain propagator carotenyl radical in the absence and presence of oxygen, respectively (Rousseau *et al.*, 1992). It is possible that carotenoids protect cells at low concentration but increase the extent of damage at higher concentrations (Lowe *et al.*, 1999). It is also possible that carotenoids may protect cells when levels of other antioxidant vitamins are low, while low levels of circulating micronutrients may enhance the susceptibility of tissue to oxidative damage levels required to offer protective effect may be higher than the physiological levels or the levels prescribed as Recommended Daily Allowances (RDA). Intake of non-nutrients through diet may therefore be considered to prevent tissue damage and overcome marginal deficiencies in micronutrient vitamin levels. This study suggests the protective antigenotoxic role of ginger, which is a commonly used condiment.

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