RESEARCH COMMUNICATION

Dietary Cardamom Inhibits the Formation of Azoxymethane-induced Aberrant Crypt Foci in Mice and Reduces COX-2 and iNOS Expression in the Colon

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Abstract

Recently, considerable attention has been focused on identifying naturally occurring chemopreventive compounds capable of inhibiting, retarding, or reversing the multi-step carcinogenesis. The primary aim of the present study was to identify the effects of a commonly consumed spice, viz., cardamom against azoxymethane (AOM) induced colonic aberrant crypt foci (ACF) in Swiss Albino mice. The secondary aim, was to explore the ability of cardamom to modulate the status of proliferation and apoptosis, and to understand its role in altering cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression. Male Swiss albino mice were injected with AOM (dose: 5mg/Kg body weight) or saline (Group 1) weekly once for two weeks. The AOM-injected mice were randomly assigned to two groups (Groups 2 and 3). While all the groups were on standard lab chow, Group 3 received oral doses of 0.5% cardamom, in aqueous suspension, daily for 8 weeks. Following treatment, significant reduction in the incidences of aberrant crypt foci (p<0.05) was observed. This reduction in ACF was accompanied by suppression of cell proliferation (mean Brdu LI in carcinogen control=13.91±3.31, and 0.5% cardamom=2.723±0.830) and induction of apoptosis (mean AI in carcinogen control=1.547±0.42 and 0.5% cardamom= 6.61±0.55). Moreover, reduction of both COX-2 and iNOS expression was also observed. These results suggest that aqueous suspensions of cardamom have protective effects on experimentally induced colon carcinogenesis. Cardamom as a whole and its active components require further attention if the use of this spice is to be recommended for cancer prevention.

Key Words: Cardamom - azoxymethane - aberrant crypt foci - COX-2 - iNOS

Introduction

Colorectal cancer is still a leading cause of cancer deaths in the United States and is increasing at an alarming rate in developing countries due to changing dietary habits and lifestyle (Greenlee et al., 2000; Yeole et al., 2001). Plants, vegetables, herbs and spices used in folk and traditional medicine are currently being recognized as cancer chemopreventive agents (Gupta, 2001); because of their antioxidant and anti-inflammatory properties that plausibly contribute to their anticarcinogenic and antimutagenic activities (Ann et al., 2001). Some of them have many other beneficial effects like hypocholesterolaemic, hypoglycaemic, and antimicrobial properties (Krishnaswamy & Polasa, 2001).

*Elettaria cardamomum* [Family.Zingiberaceae]. Cardamom, is a sweet spice and is employed as a medicinal flavouring agent. Cardamom has been reported to possess antioxidant properties. The existing use of this spice as an antioxidant and anti-inflammatory agent in traditional and folklore medicine, prompted us to use it as treatment agent in our experiment.

The present study was designed to observe the inhibiting effect of aqueous suspension of cardamom on azoxymethane (AOM) induced colon carcinogenesis in Swiss Albino mice in terms of incidences of ACF. Enhancement of cell proliferation, and inhibition of apoptosis are considered to be the risk factors for tumour development as well as for multistep carcinogenesis in colon (Jenab et al., 2001). Thus, in the present study the ability of cardamom suspension to modulate the level of cell proliferation and apoptosis, was assessed.

Cyclooxygenase (COX)-1 and COX-2 are isoforms responsible for the production of prostaglandin (PG) from arachidonic acid. While COX-1 is constitutive .COX-2 is the inducible form and is overexpressed in colon tumors (Ferrandez et al., 2003; Ota et al., 2002). Similarly, inducible nitric oxide synthase (iNOS), a generator of cellular nitric
oxide is also overexpressed in colon tumors (Barrachina et al., 2001; Watanabe et al., 2000). It is well known that the inhibition of COX-2 and iNOS activities lead to the prevention and/or control of colon cancer (Watanabe et al., 2000; Ricchi et al., 2003). Thus, in this study we also have aimed to test if suspension of cardamom modulates the expression of COX-2 or iNOS proteins.

**Materials and Methods**

Male Swiss albino mice, (obtained from animal colony of our Institution) were maintained in plastic cages (~6 mice / cage) at an ambient temperature of 22-25°C on a 12 hour light / dark cycle with access to drinking water and pellet diet (Lipton India Ltd) ad libitum. Use of animals was under strict animal care ethics guidelines of the institute.

AOM (Sigma Chemicals Co. MO, USA) was injected intraperitoneally once a week for two weeks (5mg/kg b. w.). Cardamom was bought from local market. The spice was powdered in a mixer and diluted with distilled water so as to make a 0.5% (w/v) aqueous suspension of Cardamom. The suspensions were prepared fresh everyday before oral administration at a dose of 100µl / mouse per day. In our previous studies (unpublished), we used three doses, viz. 0.25%, 0.5% and 1% (w/v) solutions of cardamom in order to observe their effects on ACF formation during AOM induced colon carcinogenesis. Treatment with the selected dose (0.5% (w/v) aqueous suspension of Cardamom) was found to be most effective in reducing the incidences of ACF without any toxic manifestations.

**Experimental Design**

The experiment was designed in four sets according to the experimental parameters used. Each set, having 30 mice, was divided into 3 groups - Normal, Carcinogen Control and Cardamom treated groups. Each group in a set consisted of 10 mice. All the mice were injected with azoxymethane (AOM, dose: 5mg/Kg body weight) or saline (Group-1), weekly once for two weeks. The AOM-injected mice were randomly assigned to two groups (Groups2 and 3). While all the groups were on standard lab chow, Group 3 received oral dose of 0.5% cardamom, in aqueous suspensions, respectively; daily for 8 weeks. The treatment was commenced from the first day of AOM injection in Group 3. The different parameters were studied thereafter.

**Set I: Assessment of ACF**

After 8 weeks of 1st AOM injection, the mice in this set were sacrificed to assess the incidences of colonic AOM, identified, by staining with 2% methylene blue (Qualigens Ltd.), on glass slides with mucosal side up under the light microscope at 100X magnification, by their large and elliptical luminal opening (Mclellan et al., 1991; Bird, 1987) .The total number ACF was counted for each mouse.

**Set II: Detection of In situ Cell Proliferation**

Cell proliferation in colon was measured using 5-bromo-2’-deoxyuridine labelling (Davidson et al., 2000) and a Detection Kit II, procured from Roche Molecular Biochemicals. The Proliferative Index (PI) was determined by dividing the number of labelled cells by the total cells counted and multiplying by 100.

**Set III: In situ Cell Death detection (Apoptosis)**

Apoptotic cells in colon were visualized using the terminal deoxynucleotidyl transferase (TdT) - mediated dUTP- biotin nick end labelling (TUNEL) method with the help of in situ cell death detection kit, AP (Roche Molecular Biochemicals). Apoptotic Index (AI) was determined as the percentage of the labelled nuclei with respect to the total number of nuclei counted (Cadermi et al., 2000).

**Set IV: SDS-PAGE Western blotting for COX-2 and iNOS Expression**

Distal 5cms of colon tissues were removed, washed in PBS at 0˚C, cut into pieces and homogenized in 5 volumes of ice-cold homogenizing buffer (0.1M NaCl, 0.01M Tris-Cl, 0.001M EDTA) containing 1mM PMSF, 1µg/ml Aprotinin, 0.1 mM Leupeptin. After centrifugation at 13000 rpm for one hour at 4˚C, the supernatants were estimated for their protein content using Bovine Serum Albumin (BSA) as a standard (Lowry et al., 1951).

SDS-PAGE and Western blotting were carried out essentially as described by Laemml. (1970). Two separate gels, one 10% (for COX-2) and another 8% (for iNOS) were run simultaneously. Electrophoretically resolved proteins were transblotted onto Immobilon-P (PVDF) membranes and subsequently blocked with 5% TBS blotto A (Santa Cruz Biotech. Inc., Santa Cruz, California) diluted 1:500 in TBS containing 0.5% TBS blotto A (non fat dried milk). After extensive washing, blots were reincubated with HRP-conjugated anti-rabbit secondary antibody (Santa Cruz Biotech. Inc., Santa Cruz, California) and incubated with the anti-COX-2 and anti-iNOS rabbit polyclonal antibodies (Santa Cruz Biotech. Inc., Santa Cruz, California) diluted 1:500 in TBS containing 0.5% TBS blotto A (non fat dried milk). After extensive washing, blots were reincubated with HRP-conjugated anti-rabbit secondary antibody (Santa Cruz Biotech. Inc., Santa Cruz, California). The protein bands were then visualized using Luminol reagent (Santa Cruz Biotech. Inc. Santa Cruz, California) and applied for exposure of X-ray film.To demonstrate equal loading of samples onto the gel, the PVDF membranes were stained with Coomassie Brilliant blue (Tao et al., 2002).

**Statistical Analysis**

The differences in mean values among different groups were tested and the values were expressed as mean ± SD. Data obtained from incidences of ACs, AI and BrdU LI were analyzed using Students-t-test. All the statistical calculations were carried out using Microsoft Excel and a P-value < 0.05 was considered significant.

**Results**

**ACF**

At the end of 8 weeks, in comparison to the carcinogen controls, total number of ACF was significantly lower by
48.33 % in the cardamom (p<0.005) treated group (Table 1). Mean numbers of ACF, consisting of 4 or more aberrant crypts in each group, are also presented in Table I. The numbers of foci consisting of 4 or more crypts were significantly lower (p<0.001) in mice treated with aqueous suspension of cardamom. No aberrant crypts were visible in the colons of normal mice.

**Cell Proliferation and Apoptosis**

Sections from distal 5cm of colons were chosen and the cells were counted. Significant reduction in Brdu LI (Figure 1) and significant induction in AI (Figure 2) was observed in the treatment group. The Brdu LI increased significantly in the carcinogen control group (13.91±3.31; p<0.001) from that observed in the normal group (2.91±0.33). Cardamom significantly reduced Brdu LI (2.72±0.83) in comparison to the carcinogen control group (p<0.001).

AI was found to decrease in the carcinogen control group (1.54±0.421; p<0.001) with respect to the normal group (2.99±0.603). Following treatment, the AI increased significantly in 0.5% cardamom (6.61±0.55) as compared to the carcinogen control group (p<0.001).

**Cyclooxygenase-2 Expression**

Expression of COX-2 protein in Normal, Carcinogen control and Cardamom treated animals has been represented in Figure 3. COX-2 immunoreactivity with polyclonal anti-COX-2 antibody was not detectable in colonic fraction of Normal mice. In contrast, dark immuno reactive bands of COX-2 at a position of 72KD were observed in animals treated with AOM only (Carcinogen control). In case of the cardamom treated animals, all the lanes showed negligible expression of COX-2.

**Inducible Nitric Oxide Synthase Expression**

Data for expression of iNOS are presented in Figure 4. As with COX-2 expression, there was no detectable iNOS immunoreactivity with polyclonal anti-iNOS antibody in colonic fractions of normal mice. Whereas immunoreactive bands at a position of 117KD were clearly observed in carcinogen control mice, they were only very faint in the treated group.

**Discussion**

The major aim of the present study was to elucidate the inhibitory role of cardamom against AOM induced colonic
involvement of COX-2 and iNOS in colon carcinogenesis compared to the carcinogen control group. As the cardamom was able to reduce the expression of iNOS as determined by Western Blot analysis in our experiment.

In conclusion, our preliminary study on reduced risk of colon cancer associated with dietary consumption of cardamom is of great importance particularly in relation to the global prevalence of this cancer. This beneficial spice, therefore, is worthy of serious consideration for further study. Future studies should me aimed at elucidating the metabolism, pharmacokinetics, and toxicity of the active compounds of cardamom. Clinical trials could be useful for assessing the ability of cardamom to prevent human colon cancer or pre-neoplastic lesions.

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