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Effect of kaempferol on lipid peroxidation and antioxidant status in 1,2-dimethyl hydrazine induced colorectal carcinoma in rats

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ABSTRACT

Colorectal cancer, a common cause of cancer related deaths in both sexes in western population is often due to persistent oxidative stress leading to DNA damage. Antioxidants scavenge free radicals and inhibit neoplastic process. Kaempferol, a flavonol widely distributed in tea, broccoli, grape fruit, brussel sprouts and apple and is claimed to have chemopreventive action in colon cancer. The aim of our study was to evaluate the effect of kaempferol on tissue lipid peroxidation and antioxidant status in 1,2-dimethyl hydrazine induced colorectal cancer in male wistar rats and to compare its efficacy with irinotecan. Experimental colon cancer induced by 1,2-dimethyl hydrazine in rats mimic human colon cancer and therefore is an ideal model for chemoprevention studies. The rats were divided into six groups. Group 1 served as control. Group 2 received 1,2-dimethyl hydrazine (20 mg/kg body weight) subcutaneously once a week for four weeks. Group 3 received irinotecan (100 mg/kg body weight) intravenously once a week for four weeks with 1,2-dimethyl hydrazine. Groups 4 to 6 were given a daily oral dose of 50, 100, 200 mg/kg body weight of kaempferol with 1,2-dimethyl hydrazine. The total study period was 16 weeks. Kaempferol supplementation lowered 1,2-dimethyl hydrazine induced erythrocyte lysate and liver thiobarbituric acid reactive substances level and rejuvenated anti oxidant enzymes catalase, super oxide dismutase and glutathione peroxidase. The recovery of enzyme status was maximum at the dose of 200 mg/kg body weight and was comparable to irinotecan. Our study reveals that kaempferol could be safely used as a chemopreventive agent in colorectal cancer.

1. Introduction

Colorectal cancer, a common cause of cancer related deaths in both sexes in western population is often due to persistent oxidative stress resulting in DNA damage and inhibition of tumor suppressor gene (Goel et al., 2001). Familial adenomatous polyposis is characterized by hundreds of colorectal adenomas or polyps which eventually transform into colon cancer. In about 80% of cases, colorectal cancer is due to improper diet (Franco et al., 2005) and hence may be prevented by dietary modifications. Several studies have reported that diet rich in fruits and vegetables markedly decrease the risk of colorectal cancer. Risk reduction by nutritional intervention may provide an alternate approach in secondary prevention of cancer (Slattery et al., 1999). The purpose of the study is to explore the possibility of such an approach.

Bioflavonoids, the phytochemicals present in abundant quantities in a variety of plants play a vital role in cancer prevention (Miean and Suhaila Mohamed, 2001) as they have the ability to scavenge free radicals (Agarwal and Nagarathinam, 1981). Kaempferol, a flavonol widely found in tea (Park et al., 2006), broccoli, grape fruit, brussel sprouts and apple and is claimed to have an anti proliferative effect on colon cancer cell lines (Li et al., 2009). The anti-inflammatory (Lee et al., 2007), antiangiogenic (Luo et al., 2009) properties of kaempferol have also been well documented. Among the flavonols, kaempferol is absorbed well when administered orally, even in low doses with minimal inter individual variation (Dupont, 2004).

1,2-dimethyl hydrazine, a potent carcinogen with selectivity for colon can produce colon cancer in experimental rats. Being a procarcinogen, it requires metabolic activation in the liver to become an active carcinogen. It is an alkylating agent and its hydroxyl metabolite methylazoxymethanol results in the formation of methyldiazonium ions that alkylate DNA, RNA and protein. Alkylation of oxygen atoms of purine and pyrimidine bases of DNA, has been suggested to lead to mutagenesis and carcinogenesis (Hawks and Magee, 1974). 1,2-dimethyl hydrazine is also metabolized into a methyl free radical and generates hydrogen peroxide in the presence of metal ions that may contribute to initiation of lipid peroxidation (Dudeja and Brasitus, 1990). Reactive oxygen species, due to their higher reactivity are potentially toxic, mutagenic or carcinogenic (Nordberg and Arner, 2001) and lead to development of oxidative stress on different cells including red blood cells.

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Table 1
Effect of kaempferol on thiobarbituric acid reactive substances in erythrocyte lysate, liver and colon.

Groups	Eyrthrocyte lysate	Liver	Colon
	(pmol/mg Hb)	(mmol/mg of tissue)	
Control	1.867 ± 0.180^{a}	0.501 ± 0.048^{a}	0.449 ± 0.043^{a}
DMH*	6.579 ± 0.633^{b}	$1.027 \pm 0.0.099^{b}$	0.109 ± 0.010^{b}
Irinotecan	1.943 ± 0.187^{a}	0.659 ± 0.063^{c}	0.330 ± 0.032^{c}
Kaempferol 50 mg/kg	4.575 ± 0.440^{d}	0.845 ± 0.082^{d}	0.468 ± 0.041^{a}
Kaempferol 100 mg/kg	4.468 ± 0.430^{d}	0.667 ± 0.054^{c}	0.370 ± 0.039^{c}
Kaempferol 200 mg/kg	3.329 ± 0.340^{c}	0.536 ± 0.043^{a}	0.321 ± 0.022^{c}

All the values are expressed as mean ± S.D. of 6 rats in each group. Values that have a different superscript letter (a,b,c,d) differ significantly with each other ($P<0.05$, DMRT).
*1,2-dimethylhydrazine.

1,2-dimethyl hydrazine induces cell proliferation resulting in hyper cellularity of crypts and development of foci of atypical epithelium. These aberrant crypts precede the appearance of dysplastic crypts followed by microadenoma, adenoma and adeno carcinoma. Experimental colon cancer induced by 1,2-dimethyl hydrazine in rats is of epithelial origin, morphologically and histologically similar to human colon cancer and therefore is an ideal model for chemoprevention studies. The aim of the present study is to evaluate the effect of kaempferol on lipid peroxidation in liver, colon and erythrocyte lysate and antioxidant enzymes like superoxide dismutase, glutathione peroxidase and catalase in liver and colon in 1,2-dimethyl hydrazine induced colon cancer and also to compare its efficacy with irinotecan, a well known anti cancer agent used in the treatment of colon cancer.

2. Materials and methods

2.1. Chemicals and carcinogen

1,2-dimethyl hydrazine (DMH) and kaempferol were purchased from Sigma Chemical Company (St Louis, MO, USA). All other chemicals and reagents used were of analytical grade. 1,2-dimethyl hydrazine was dissolved in 1 mM EDTA just prior to use and the pH was adjusted to 6.5 with 1 mM NaoH.

2.2. Preparation of drug

Kaempferol powder was used as a suspension in 0.1% carboxymethyl cellulose and each rat received a daily dose of 50,100 and 200 mg/kg body weight of kaempferol in 2 ml of suspension.

2.3. Animals and experimental design

36 male wistar rats (n = 6 per group) were used in this study. 1,2-dimethyl hydrazine was given at the dose of 20 mg/kg body weight s.c. once a week for four consecutive weeks (Deeptha et al., 2006). Irinotecan was given in the dose of 100 mg/kg body weight i.v. once a week for four consecutive weeks (Cao and Rustum, 2000).

The study was started after getting approval from the Institutional Animal Ethical Committee. The rats were housed in the animal house, Rajah Muthiah Medical College, Annamalai University in an air conditioned room with a 12 h light and dark cycle. The animals were provided with vitamin enriched pellet diet consisting of 23% wheat flour, 60% roasted Bengal gram powder, 5% skimmed milk powder, 4% casein, 4% refined oil, salt mixture with 4% starch and choline. The rats were maintained in accordance with the Indian national law on animal care and use (Reg. No. 190/2007/CPCSEA).

Group 1 (n = 6)	Control	Rats received pellet diet for 16 weeks and served as control.
Group 2 (n = 6)	DMH	Rats received pellet diet for 16 weeks and were administered 1,2-dimethyl hydrazine (20 mg/kg) s.c. once a week for 4 weeks.
Group 3 (n = 6)	Irinotecan	Rats received pellet diet for 16 weeks and were administered 1,2-dimethyl hydrazine (20 mg/kg) s.c. and irinotecan (100 mg/kg) i.v. once a week for 4 weeks.
Group 4 (n = 6)	Test group 1	Rats received pellet diet along with kaempferol in the dose of 50 mg/kg through oral gavage for 16 weeks and were administered 1,2-dimethyl hydrazine (20 mg/kg) s.c. once a week for 4 weeks.
Group 5 (n = 6)	Test group 2	Rats received pellet diet along with kaempferol in the dose of 100 mg/kg through oral gavage for 16 weeks and were administered 1,2-dimethyl hydrazine (20 mg/kg) s.c. once a week for 4 weeks.
Group 6 (n = 6)	Test group 3	Rats received pellet diet along with kaempferol in the dose of 200 mg/kg through oral gavage for 16 weeks and were administered 1,2-dimethyl hydrazine (20 mg/kg) s.c. once a week for 4 weeks.

The total period of study was 16 weeks. The weights of the rats were recorded at the beginning of the experiment, at weekly intervals and at the end of study period. The animals were sacrificed after overnight fasting at the end of 16 weeks.

2.4. Blood collection and preparation of erythrocyte lysate

After sacrificing the rats, blood was collected into 5.0 ml heparinized tubes and plasma was separated by centrifugation at 800 × *g* for 5 min at 4 °C. After separation of plasma, the buffy coat was removed and packed cells were washed thrice with cold physiological saline containing glucose (5.5 mM).

Erythrocyte lysate was prepared by lysing a known volume of erythrocytes by addition of two volumes of distilled water to packed erythrocytes and were centrifuged at 3000 × *g* for 10 min at 4 °C to separate the erythrocyte lysate.

Erythrocytes are vulnerable targets for oxidative stress due to their high intracellular concentration of iron and the presence of numerous double bonds in the membrane bound polyunsaturated fatty acids (Glass and Gershon, 1984). Highly reactive hydroxyl radicals are

Table 2
Effect of kaempferol on catalase and super oxide dismutase in liver and colon.

Groups	Catalase		Super oxide dismutase	
	(μmol of H_2O_2 utilized/min/mg protein)		(enzyme required for 50% inhibition of NBT reduction/min/mg protein)	
	Liver	Colon	Liver	Colon
Control	56.221 ± 5.412^{a}	64.812 ± 6.239^{a}	7.40 ± 0.60^{a}	3.81 ± 0.33^{a}
DMH*	23.884 ± 2.299^{b}	30.204 ± 2.908^{b}	3.20 ± 0.30^{b}	2.30 ± 0.19^{b}
Irinotecan	$52.201 \pm 5.025^{a,c}$	58.670 ± 5.647^{c}	7.31 ± 0.72^{a}	3.70 ± 0.25^{a}
Kaempferol (50 mg/kg)	45.468 ± 5.242^{d}	42.544 ± 4.095^{e}	3.30 ± 0.11^{b}	2.51 ± 0.18^{c}
Kaempferol (100 mg/kg)	48.066 ± 4.627^{c}	53.570 ± 4.562^{d}	3.82 ± 0.22^{c}	$2.40 \pm 0.16^{b,c}$
Kaempferol (200 mg/kg)	55.488 ± 5.341^{a}	62.694 ± 6.035^{a}	6.14 ± 0.31^{d}	3.16 ± 0.18^{d}

All the values are expressed as mean ± S.D. of 6 rats in each group. Values that have a different superscript letter (a,b,c,d) differ significantly with each other ($P<0.05$, DMRT).
*1,2-dimethylhydrazine.

generated through the interaction of iron and reactive oxygen species which result in molecular damage (Masotti et al., 1988). Thus, erythrocytes are excellent targets for the study of oxidative stress in carcinogenesis. The concentration of thiobarbituric acid reactive substances in the liver and erythrocyte lysate reflect endogenous lipid peroxidation and are elevated in carcinogen exposed animals (Manju and Nalini, 2005).

2.5. Preparation of tissue homogenate

The rat colon was scraped gently with ice cold microscope slide and scrapings of the mucosa were collected. Colonic mucosal scrapings and liver sections were homogenized in an ice cold phosphate buffer of pH 7.0, centrifuged and used for assays.

2.6. Estimation of antioxidant enzyme activity and thiobarbituric acid reactive substances

Thiobarbituric acid reactive substances in liver and colon were estimated by the method of Okhawa (Ohkawa et al., 1984) and thiobarbituric acid reactive substances in erythrocyte lysate by the method of Yagi (Yagi, 1984). The level of super oxide dismutase was estimated in liver and colon by the method of Kakkar (Kakkar et al., 1984). Catalase was assayed through the method of Sinha (Sinha, 1972) and its activities were measured in both liver and colon. The levels of reduced glutathione and glutathione peroxidase in liver and colon were determined by the methods of Boyne and Ellman (Boyne and Ellman, 1972) and Folhe and Gunzler (Fohle and Gunzler, 1984) respectively. The results were compared with irinotecan group.

2.7. Statistical analysis

The statistical significance of the data was determined using one way analysis of variance (ANOVA) and the significant difference between the treatment groups were evaluated by Duncan's Multiple Range Test (DMRT). The results were considered as statistically significant at $P<0.05$.

3. Results

3.1. General observation

There was no statistically significant difference in the final body weight (g), body weight gain/loss (g), food intake (g/day) and food efficiency (body weight gain [g/day]/food intake) among the experimental groups (data not shown). No signs of toxicity were observed in any of the groups during the entire study period.

3.2. Effect on lipid peroxidation product

Erythrocyte lysate and liver thiobarbituric acid reactive substances were significantly elevated ($P<0.05$) while colonic mucosal thiobarbituric acid reactive substances was significantly reduced ($P<0.05$) in 1,2-dimethyl hydrazine treated group (group 2). Kaempferol supplementation in the dose of 50 mg/kg with 1,2-dimethyl hydrazine (group 4) altered this level. Erythrocyte lysate and liver thiobarbituric acid reactive substances were reduced and colonic thiobarbituric acid reactive substances increased significantly in this group. However supplementation of kaempferol at the dose of 200 mg/kg with 1,2-dimethyl hydrazine (group 6) lowered the level of erythrocyte lysate and liver thiobarbituric acid reactive substances and elevated colonic mucosal thiobarbituric acid reactive substances more effectively. The changes in the levels of thiobarbituric acid reactive substances were more apparent in group 6 (kaempferol 200 mg/kg) than in group 5 (kaempferol 100 mg/kg) (Table 1).

3.3. Effect on catalase and super oxide dismutase

The antioxidant enzymes super oxide dismutase and catalase were significantly decreased in 1,2-dimethyl hydrazine treated group (group 2) when compared to control group (group 1). However kaempferol supplementation restored the level of these enzymes to near normal values in a dose dependent manner. The levels of super oxide dismutase and catalase in group 6 (kaempferol 200 mg/kg) were comparable to group 1 (control group) (Table 2).

3.4. Effect on glutathione peroxidase and reduced glutathione

Glutathione peroxidase and reduced Glutathione along with catalase and superoxide dismutase form the primary anti oxidant defense. The levels of liver and colonic glutathione peroxidase and reduced glutathione were significantly reduced in 1,2-dimethyl hydrazine treated group (group 2) when compared to control group (group 1). Kaempferol supplementation at the dose of 50 mg/kg and 100 mg/kg (groups 4 and 5) rejuvenated these anti oxidant enzymes and kaempferol at the dose of 200 mg/kg (group 6) further increased their levels (Table 3).

4. Discussion

Free radicals play a pivotal role in the pathogenesis of colorectal cancer and are aggressive substances that bring about cell damage and cell death. A cell can tolerate the toxic effects of small amounts of free radicals that are produced during the course of normal metabolism. However, when free radical production is enormously increased as in pathological states it results in cell damage (Nakagami et al., 1999). The data in our study reveal that there is an inverse relationship between lipid peroxidation and the rate of cellular proliferation i.e., the highly proliferating tumor cells have shown notably low level of lipid peroxidation products as compared to normal cells. The levels of

Table 3
Effect of kaempferol on glutathione peroxidase and reduced glutathione in liver and colon.

Groups	Glutathione peroxidase (μmol of GSH utilized/min/mg protein)		Reduced glutathione (mg/g of tissue)	
	Liver	Colon	Liver	Colon
Control	7.479 ± 0.720^{a}	6.786 ± 0.653^{a}	26.520 ± 2.553^{a}	27.200 ± 2.618^{a}
DMH*	3.646 ± 0.351^{b}	3.259 ± 0.314^{b}	15.980 ± 1.538^{b}	14.484 ± 1.394^{b}
Irinotecan	$7.159 \pm 0.689^{a,c}$	$6.057 \pm 0.583^{c,d}$	24.548 ± 2.363^{a}	24.648 ± 2.376^{a}
Kaempferol (50 mg/kg)	5.943 ± 0.572^{d}	$5.825 \pm 0.561^{c,d}$	21.828 ± 2.101^{c}	21.420 ± 2.062^{c}
Kaempferol (100 mg/kg)	5.920 ± 0.570^{d}	6.124 ± 0.576^{c}	22.941 ± 1.841^{c}	22.369 ± 1.841^{c}
Kaempferol (200 mg/kg)	$7.209 \pm 0.694^{a,c}$	$6.431 \pm 0.619^{a,c}$	25.840 ± 2.487^{a}	26.520 ± 2.553^{a}

All the values are expressed as mean ± S.D. of 6 rats in each group. Values that have a different superscript letter (a,b,c,d) differ significantly with each other ($P<0.05$, DMRT).
*1,2-dimethylhydrazine.

thiobarbituric acid reactive substances in erythrocyte lysate and liver were significantly increased whereas its level in colonic tissue was significantly reduced in 1,2-dimethyl hydrazine treated rats. The tumor cells exhibit a distinctly low level of lipid peroxidation when compared to their normal counterparts (Diplock et al., 1994). Cellular proliferation appears to be rapid whenever the lipid peroxidation is low (Schmelz et al., 2000). Besides, tumor cells successfully ward off free radical attack. Moreover lipid peroxidation level found in the colon of 1,2-dimethyl hydrazine treated rats correlates with increased cellular proliferation. Kaempferol supplementation resulted in significant alteration of this level. Thiobarbituric acid reactive substances in erythrocyte lysate and liver were significantly decreased and that of colonic tissue was increased in a dose dependent manner, signifying the recovery phase. The most significant effect of kaempferol supplementation was seen at the dose of 200 mg/kg body weight and it was comparable with irinotecan group.

Superoxide dismutase plays an important role in scavenging super oxide anions which are formed during the early stages of oxidative stress. Catalase catalyzes the formation of water and oxygen from hydrogen peroxide and prevents oxidative damage (Rajeshkumar and Ramadasan, 2003). Both these enzymes limit the effect of oxidant molecules on tissues and are activated during oxidative cell injury (Kyle et al., 1987). Together they act synergistically to eliminate reactive oxygen species and a small deviation in their physiological concentration may have a dramatic effect in the protection of cells against oxidative damage (Mates and Sanchez, 1999). The active metabolite of 1,2-dimethyl hydrazine induces colon cancer by promoting oxidant damage to DNA, protein and other macromolecules. In our study 1,2-dimethyl hydrazine treatment resulted in decreased colonic superoxide dismutase and catalase activities. The decreased activities of superoxide dismutase and catalase in colon are due to a natural cellular response against reduced level of reactive oxygen species (Giftson et al., 2010).

One of the most abundant intracellular thiols, reduced glutathione and glutathione peroxidase protect cell from the lethal effect of toxic substances (Gopalan et al., 1992). In the presence of 1,2-dimethyl hydrazine, glutathione peroxidase is induced as detoxifying enzyme and this results in the conjugation of toxic electrophiles with reduced glutathione (Dani et al., 2007). Induction of such enzymes determines the potency of many anticancer agents (Singh et al., 2006). Kaempferol rejuvenated and restored these antioxidant enzymes, thus acting as an ideal chemopreventive agent. Recovery of antioxidant enzymes status was best seen with kaempferol supplementation at the dose of 200 mg/kg body weight.

Chemoprevention *per se* was accepted as a form of cancer cure only after Chemoprevention Working Group submitted its report to the American Association of Cancer Research. This report marked a new era in the management of cancer. Kaempferol has been reported to be effective in pancreatic (Nothlings et al., 2007) and lung cancers (Leung et al., 2007) and also in human glioma (Jeong et al., 2009). It is claimed to act as a natural antioxidant and free radical scavenger minimizing the damage inflicted to plasma membranes and genes. It has been reported to act synergistically with quercetin to bring about a significant reduction in cell proliferation in human gut cell lines (HuTu080 and Caco-2) and breast cancer cells (Margaret et al., 2005).

At present irinotecan, a semisynthetic analogue of the plant alkaloid camptothecin is extensively used in the treatment of colorectal, esophageal, gastric, non-small cell and small cell lung cancers. It is a topoisomerase 1 inhibitor. Its active metabolite SN-38 leads to inhibition of DNA replication and transcription. But, major side effects of irinotecan are life threatening diarrhoea and immunosuppression. It is currently used as one of the first line drugs in combination with 5-Flurouracil and leucovorin in the management of colorectal carcinoma. Multi drug resistance of irinotecan is a major problem encountered in cancer chemotherapy. It is due to over expression of ATP binding cassette transmembrane transporters like P-glycoprotein or multi drug resistance associated protein which causes the efflux of drugs from cancer cells (Xu and Villalona-calero, 2002).

Kaempferol, also a plant product has the following advantages over irinotecan. It can be given orally, is absorbed well and the bioavailability is proportionate to its dose (Barve et al., 2009). In contrast, irinotecan can be given only parenterally. Being a dietary component available in many plant sources, kaempferol can be used as a chemopreventive agent in high risk individuals with familial adenomatous polyposis. Irinotecan can never be used as a chemoprophylactic agent. The maximum tolerated dose of kaempferol is 1700 mg/m^2 thrice weekly and no serious adverse drug reactions have been reported so far. Moreover kaempferol has been reported to inhibit the P-glycoprotein or multi drug resistant protein-1 mediated drug efflux and so can overcome the drug resistance encountered in cancer cells (Hooijberg et al., 1999).

5. Conclusion

Our study reveals that kaempferol can be a potential chemopreventive agent in colorectal cancer. It is most effective at the dose of 200 mg/kg and its action is comparable to that of irinotecan, the first line drug used in the management of colorectal cancer. Kaempferol is safe, devoid of serious adverse effects, can overcome drug resistance and can be used as a chemopreventive agent.

Conflict of interest

There is no conflict of interest to be disclosed.

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