Chapter-VII

Inhibitory effects of rutin against 1,2-dimethylhydrazine induced aberrant crypt foci, mucin depleted foci and colon tumorigenesis in Wistar rats
1. Background

Chemoprevention has emerged as a pragmatic and translational approach to reduce the incidences and mortality from various cancers including colon cancer (Rajamanickam et al. 2010). The goal of cancer chemoprevention is to slow, block, or reverses the process of carcinogenesis through the use of natural or synthetic compounds (Johnson and Mukhtar 2007). CRC is the 3rd most common malignancy and 4th most common cause of cancer mortality worldwide (Tenesa and Dunlop 2009). Colon cancer is one of the most common, best-understood tumors from a genetic point of view, yet it remains the leading cause of cancer-related mortality in men and women (Brien et al. 2007, Jemal et al. 2009). It thought to arise by the accretion of genetic alterations involving a variety of oncogenes and tumor suppressor genes that transform normal colonic epithelium into an invasive carcinoma, with aberrant crypt foci (ACF) and mucin depleted foci (MDF) as putative preneoplastic lesions in this transformation process (Janne et al. 2000; Khan et al. 2011; Mori et al. 2005). ACF were first discovered in the colon of carcinogen treated rodent (Bird et al. 1987) and they have also been observed in patients with sporadic colorectal cancer (CRC) and with familial adenomatous polyposis (FAP) (Pretlow et al. 1991; Roncucci et al. 1991; Nucci et al. 1997). They are identified by their elevated crypts, thicker colonic epithelial cell lining and increased pericryptal zone comparative to normal crypts (Bird et al. 1987). It has already been reported that ACF have been used as intermediate biomarkers or short term bioassays to identify modulators of colon carcinogenesis in many chemopreventive studies (Kawamori et al. 1994; Rao et al. 1993a; Pereira and Khoury 1991; Pereira et al. 1994; Rao et al. 1993b; Thorup et al. 1994; Takahashi et al. 1993). MDF is another preneoplastic lesion of colon cancer and they are formed by dysplastic crypts devoid of mucin production which is secreted by goblet cells, and they were first identified in the unsectioned colon of carcinogen treated rodents (Caderni et al. 2003). Some chemopreventive studies in rodent
models demonstrate a correlation between the presence of MDF and the development of colon cancer, and suggest that MDF might, therefore, serve as surrogate biomarkers of colon cancer (Femia et al. 2004; Femia et al. 2005; Arikawa and Gallaher 2008).

1,2-dimethylhydrazine (DMH) is an organotropie colon specific carcinogen and its repetitive administration results in the development of colonic tumors in rodent models. DMH-induced tumorigenesis in the rat colon is a prolonged multistage process, bearing many of the same cell kinetic, histopathological and molecular characteristics of tumorigenesis in the human colon (Latham et al. 1999). DMH undergoes metabolism mainly in the liver and to some extent also in the colon and the ultimate metabolite thus formed in the liver delivered to the colon via, blood or bile, as glucoronide conjugates (Oravec et al. 1986; Sengottuvelan et al. 2006). DMH is a proximate carcinogen and its needs metabolic activation to induce colon cancer. In vivo transformation of DMH results in the formation of azomethane and N-oxidation of azomethane leads to the formation of azoxymethane. Further, hydroxylation of azoxymethane leads to the formation of methyazoxymethanol which is an unstable compound and readily yields highly reactive electrophilic methylidiazonium ion. The latter leads to the formation of methyl free radicals and DMH also generate hydroxyl radical or hydrogen peroxide in the presence of metal ion which are known to elicit oxidative stress due to imbalance between the production of ROS and endogenous antioxidants (Arutjunian et al. 1997; Fiala 1977). It has been reported earlier that these ROS are mainly responsible for the damaging effects of the DMH in colonic tissue (Fiala 1977). DMH also cause covalent modification of DNA by 8-hydroxy-2’-deoxyguanosine (8-OHdG) adduct formation which is a marker of oxidative DNA damage (Park et al. 1989) and it has been well accepted that oxidative DNA damage plays an important role in carcinogenesis (Halliwell and Gutteridge 1990; Wei and Frenkel 1993).
Diet is considered as one of the major factors accounting for the variability in cancer incidence and mortality at these sites (Burstein 1993). Many experimental animal models have supported the idea that high fat diet augments the incidence of colon carcinogenesis (Bansal et al. 1978; Reddy et al. 1976; Reddy et al. 1977) whereas low fat and high fiber (present in fruits and vegetables) diet, decreases the risk of colon cancer (Hioki et al. 1997). Many plant phytochemicals (biologically active, non-nutritive compounds) are receiving considerable attention for their potential role in reducing cancer risk and has already been reported to possess chemopreventive properties against cancer (Wattenberg et al. 1979). Therefore, chemoprevention is a logical strategy to reduce the mortality from colon cancer because numerous chemopreventive agents are present in the diet (Corpet and Tache 2002).

Rutin, a quercetin-3-rutinosid or vitamin-P, is one of the flavonoid, which is found in various plants and foods especially in buckwheat, orange, onions, apples, tea and red wine (Hertog et al. 1993). Rutin is well known to exhibit multiple pharmacological activities including antibacterial, antitumor, anti-inflammatory, anti-diarrheal, antiulcer, anti-mutagenic, vasodilator, immunomodulator and inhibitor of inducible nitric oxide synthase (iNOS) gene expression (Hirose et al. 1999; Janbaz et al. 2002; Morimoto et al. 2011). Furthermore, rutin showed an inhibitory effect against membrane lipid peroxidation (Lopez-Revuelta et al. 2006). Rutin was found to have renal-protective effects via its antioxidant activities which suggest its protective role in oxidative stress-mediated diseases (Lopez-Revuelta et al. 2006; Korkmaz and Kolankaya 2010; Mahmoud 2011).

Since various flavonoids are ingested through food or supplements and rutin also have various biological properties. Therefore, we designed this study to explore the chemopreventive efficacy of rutin against DMH-induced precancerous lesions i.e., ACF and MDF in a medium term study.
as well as its chemopreventive efficacy against DMH-induced colon tumorigenesis in long term study in Wistar rats.

2. Treatment Regimen:

(A) For Medium Term Study (For ACF and MDF induction)

Group 1 (Control): Rats received basal diet along with distilled water (5ml/kg b.wt.).

Group 2 (DMH): Rats were administered with subcutaneous injection of DMH at the dose of 20mg/kg b.wt. once a week for first 5 weeks.
Group 3 (DMH + Ru) (I): Rats were administered with DMH as in Gp 2 and also fed rutin (50mg/kg b.wt. orally) every day for the first 5 weeks starting 1 week before carcinogen treatment (Initiation- I).

Group 4 (DMH + Ru) (PI): Rats were administered with DMH as in Gp 2 and also fed rutin (50mg/kg b.wt. orally) 2 days after the last injection of the carcinogen and continued till the end of the experiment (Post initiation-PI).

Group 5 (Only GA): Rats received basal diet + rutin (50mg/kg b.wt. orally) dissolved in distilled water everyday throughout the experiment.

(B) For Long Term Study (For Colon tumor induction)
Group 1 (Control): Rats received basal diet along with distilled water (5ml/kg b.wt.).

Group 2 (DMH): Rats were administered with subcutaneous injection of DMH at the dose of 20mg/kg b.wt. once a week for first 15 weeks.

Group 3 (DMH + Ru) (I): Rats were administered with DMH as in Gp 2 and also fed rutin (50mg/kg b.wt. orally) every day for the first 15 weeks starting 1 week before carcinogen treatment (Initiation-I).

Group 4 (DMH + Ru) (PI): Rats were administered with DMH as in Gp 2 and also fed rutin (50mg/kg b.wt. orally) 2 days after the last injection of the carcinogen and continued till the end of the experiment (Post initiation-PI).

Group 5 (Only GA): Rats received basal diet + rutin (50mg/kg b.wt. orally) dissolved in distilled water everyday throughout the experiment.

3. Results

(A) Medium Term Study

3.1 Effect of rutin and DMH on the development of ACF in colonic tissue

In DMH treated group (Group II), the number of ACF/colon is 73.33 ± 10 while supplementation with rutin in Group III (66.62 ± 8) and IV (59.67 ± 6) were non-significantly reduced the number of ACF. Original magnification: 10x (Fig. 1 and 2)
3.2 Effect of rutin and DMH on the development of MDF in colonic tissue

In DMH treated group (Group II), the number of MDF/colon is 4.89 ± 1.014 while supplementation with rutin in Group III (3.91 ± 0.464) reduced the number of MDF but at the non-significant level as compared to Group II. In Group IV, treatment with rutin significantly reduced the number of MDF (3.02 ± 0.477) (P< 0.05) as compared to Group II. Original magnification: 10x (Fig. 3 and 4)

(B) Long Term Study

3.3 Effects of DMH and rutin on tumor incidence and multiplicity.

Representative photomicrographs of morphological appearance of tumors are shown in Fig. 5. Most of the colonic tumors were developed in the distal colon of the Wistar rats. The colonic tumor incidence [(Number of tumor-bearing rats/total number of rats in each group) X 100] and tumor multiplicity (number of tumors/tumor bearing rats) in the treatment groups are summarized in Table 1. No tumors were observed in control animals (Group I) and only rutin fed group (Group V). Significant inhibition occurred in the tumor incidence and multiplicity in the DMH+ Rutin group (Group IV) as compared to DMH treated group (Group II) while in DMH+ Rutin (Group III), there is inhibition occurred in the tumor incidence and multiplicity as compared to DMH treated group (Group II) but not at the significant level.

3.4 Effects of DMH and Rutin on colonic histopathological alteration

Representative photomicrograph of a hematoxylin and eosin stained colonic sections of control group showed normal histoarchitecture with mild inflammatory cells infiltration while DMH treated group (Group II) exhibited in situ adenocarcinoma with papillary pattern, dysplastic zone, loss of goblet cell differentiation, nuclear pseudostratification, high nucleus to cytoplasmic ratio
and massive inflammatory cells infiltration. In group III and IV, rutin supplementation reduces the severity of DMH induced colonic tumors via reducing the infiltration of inflammatory cells within the colonic tumors. Colonic sections of Group V displayed normal histology as similar to that of Group I. (Fig. 6)

4. Discussion

In this study, we have observed the chemopreventive potential of rutin against DMH-induced early markers of colon cancer (ACF and MDF) and colon tumorigenesis in Wistar rats.

Intervention studies or prospective observational epidemiological studies that use incident cancer as an end point are large, lengthy and costly. For this reason, studies with surrogate end points, biomarkers of preclinical carcinogenesis, are attractive: they are potentially smaller, shorter and less costly. Studies based on such surrogate end points are, however, inherently less informative than studies with the ‘true’ end point (e.g. incident cancer) (Schatzkin and Gail 2002).

The short term bioassays could be a very useful pre-screening tool for chemopreventive agents against colon cancer. The present study was designed to investigate the chemopreventive potential of rutin via exploiting the precancerous lesions (ACF and MDF) as biomarkers of short term bioassays. But it has been reported that there are some compounds like genistein which have the ability to suppress the ACF development (Pereira et al 1994) in the short term study but enhance the development of experimental colon cancer (Rao et al 1997). Therefore we have also assessed the chemopreventive efficacy of rutin against DMH induced colon tumorigenesis in the long term administration.
ACF exhibit preneoplastic features viz., dysplasia (Paulson et al. 2005, Jen et al. 1994, Siu et al. 1997), hyperproliferation (Bird et al. 1987, Pretlow et al. 1994, Bird 1995), K-ras mutations (Stopera et al. 1992a, Stopera et al. 1992b, Vivona et al 1993, Pretlow et al. 1993), and overexpression of c-fos (Stopera et al. 1992c), β-catenin (Paulson et al. 2005, Paulson et al. 2001) and cyclin D1 (Paulson et al. 2005). MDF also exhibit preneoplastic features viz., dysplasia (Caderni et al. 2003, Femia et al. 2007, Femia et al. 2008, Sakai et al. 2011), mutations in β-catenin (Femia et al. 2005) and Apc (Femia et al. 2007) gene, over-expression of survivin (Femia et al. 2011), COX-2, i-NOS and macrophages (Femia et al. 2009) and reduced expression of MUC2 (a mucin abundantly expressed in the normal colon) and intestinal trefoil factor, a marker of goblet cell lineage (Femia et al. 2008). While the expression of p21 and p16 (inhibitors of cyclin-dependent kinases) have been found be reduced in ACF as well as MDF (Femia et al. 2006). On the basis of above evidences, both ACF and MDF are considered to be putative early biomarkers of colon cancer.

Previously, it has been reported that various natural products or phytochemicals have been shown to inhibit the development of ACF and MDF (Leonardi et al 2010; Plate and Gallaher 2006; Kawamori et al 1995; Arikawa and Gallaher 2008). The findings of the present study corroborated with the previous findings that rutin, a phytochemical or natural product also suppresses the development of ACF and MDF in Group III and Group IV as compared to DMH treated group (Group II).

Several accumulating evidences suggest that naturally occurring plant compounds or phytochemicals have chemopreventive effects against colon cancer (Reddy et al 1997; Rao et al 1995; Deschner et al 1991; Reddy et al 1999; Tanaka et al 1997). The findings of the present
study corroborated with previous findings that rutin, a phytochemical, supplementation reduced the tumor incidence and tumor multiplicity in group III (I group) as compared to group II (DMH treated group) but not at the significant level while rutin treatment significantly ($p<0.05$) reduced the tumor incidence in group IV (PI group) as compared to group II (DMH treated group). Most of the tumors were present in the distal colon and it has been reported that the propensity of the distal colon for the development of DMH-induced colonic tumors was higher as compared to proximal colon (Barth et al 2005). These findings suggest that rutin suppresses the development of DMH induced colon tumorigenesis in Wistar rats.

Histological findings revealed that control group showed normal histoarchitecture with mild infiltration of inflammatory cells as well as normal mucosal glandular structure while DMH-treated group exhibited (Group II) exhibited *in situ* adenocarcinoma with papillary pattern, dysplastic zone, nuclear pseudostratification, high nucleus to cytoplasmic ratio and massive inflammatory cells infiltration. In group III and IV, rutin supplementation decreases the severity of colonic tumors.

It can be concluded from the findings of the present study that rutin has chemopreventive potential against DMH-induced colon carcinogenesis via suppressing the development of precancerous lesions i.e., ACF and MDF and colon tumorigenesis in the colon of Wistar rats. Further studies are warranted to explore the exact mechanism of action of rutin against DMH induced colon carcinogenesis.
Figure 1. Topographic view of Aberrant Crypt Foci (ACF) in a rat colon.

(A) (B)

Figure 1. ACF in the rat colon showing round and elongated ACF (Fig. A and B) with different crypt multiplicities. The colons were opened, stained with methylene blue and observed on a glass slide. Original magnification: 10x.

Figure 2. Effects of Rutin and DMH on the Aberrant Crypt Foci (ACF).

Figure 2. Effects of DMH and rutin on incidence of ACF per rat colon. Values are expressed as Mean ± SD. Rutin non-significantly suppressed the development of ACF in Group 3 and 4 as compared to DMH treated group (Group 2). NS- Non-significant.
Figure 3. Topographic view of Mucin Depleted Foci (MDF) in a rat colon.

Figure 3. MDF in the rat colon showing depleted mucin as indicated by arrows in the above images. The colons were opened, stained with high iron diamine (HID) and alcian blue (AB). Original magnification: 10x.

Figure 4. Effects of rutin and DMH on the Mucin Depleted Foci (MDF)

Figure 4. Effects of DMH and glycyrrhizic acid on incidence of MDF per rat colon. Values are expressed as Mean ± SD. Glycyrrhizic acid non-significantly suppressed the development of ACF in Group 3 and 4 as compared to DMH treated group (Group 2). NS- Non-significant.
Figure 5: Morphological appearance of colonic tumors

Table 1: Incidence of colonic tumors and the number of tumors per tumor-bearing rat

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. of rats</th>
<th>No. of Tumor-bearing rats</th>
<th>Tumor incidence* (%)</th>
<th>Total tumor number</th>
<th>No. of tumors/tumor-bearing rat</th>
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<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>DMH</td>
<td>8</td>
<td>8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.87&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DMH+Ru (I gp)</td>
<td>8</td>
<td>6</td>
<td>75</td>
<td>10</td>
<td>1.67</td>
</tr>
<tr>
<td>DMH+Ru (PI gp)</td>
<td>8</td>
<td>5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ru only</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>nil</td>
<td>nil</td>
</tr>
</tbody>
</table>

*(Number of tumor-bearing rats/total number of rats in each group) X 100

Values are expressed as mean ± SD

Values not sharing common superscript (a and b) are significant with each other at P<0.05.
Figure 6. Effects of rutin and DMH on histopathological type of colonic tumors

![Figure 6](image_url)

Figure 2. Photomicrographs of colonic sections depicting (A) Vehicle treated control group (Group I), (B) DMH treated group (20mg/kg b. wt.) (Group II), (C) DMH + Rutin (50 mg/kg b.wt.) (Initiation group or Group III), (D) DMH + Rutin (50 mg/kg b.wt.) (Post-initiation group or Group III), (E) Only Rutin (50 mg/kg b.wt.) (Group V). The histological sections of control group showed normal histoarchitecture with mild inflammatory cells infiltration while DMH treated group (Group II) exhibited *in situ* adenocarcinoma with papillary pattern, dysplastic zone, loss of goblet cell differentiation, nuclear pseudostratification, high nucleus to cytoplasmic ratio and massive inflammatory cells infiltration. In group III and IV, rutin supplementation attenuated the severity of colonic tumors as compared to DMH treated group. Colonic sections of Group V (only rutin treated group) displayed normal histology as similar to that of Group I (control group). Insets at the right panel show a magnified view (40x magnifications) of the insets showed at the left panel (10x magnifications).