Naringin accelerates the regression of pre-neoplastic lesions and the colorectal structural reorganization in a murine model of chemical carcinogenesis


Abstract

The aim of this study was to investigate the effect of Naringin on pre-neoplastic colorectal lesions induced by chemical carcinogen in rats. Female Wistar rats weighing 130.8 ± 27.1 g received weekly one subcutaneous injection of 1,2-dimethylhydrazine (DMH, 20 mg/kg) for 10 weeks. The animals were divided into 5 groups with 6 animals in each group. Group 1: 0.9% saline; Group 2: DMH + 0.9% saline; Group 3: DMH + Naringin (10 mg/kg); Group 4: DMH + Naringin (100 mg/kg); Group 5: DMH + Naringin (200 mg/kg). G2 and G3 showed a significant increase in ACF number, AgNOR/nucleus and mitosis compared to G1. G4 and G5 presented a significant reduction in these parameters compared to G2. The number of cells producing acidic and neutral mucins, red blood cells and the level of antioxidant minerals, such as copper, magnesium, selenium and zinc, were significantly reduced in G2 and G3, but similar in G4 and G5 compared to G1. Naringin, especially at 200 mg/kg, was effective in reducing the number of pre-neoplastic lesions in rats exposed to DMH. Some of these effects may be due to reduction in cellular proliferation and tissue levels of iron together with the recovery of antioxidant mineral levels induced by this flavonoid.

Corresponding author. Address: Department of General Biology, Federal University of Viçosa, 36570-000 MG, Brazil. Tel.: +55 31 3899 2515; fax: +55 31 3899 2549. E-mail address: romuonovaes@yahoo.com.br (R.D. Novaes).

Article info

Article history:
Received 20 May 2013
Accepted 22 November 2013
Available online 1 December 2013

Keywords:
Flavonoid
Large intestine
Pathology
Toxicology
1,2-Dimethylhydrazine

1. Introduction

Cancers of the digestive tract have a high incidence among populations and are common causes of death from cancer worldwide (Pierini et al., 2008). Due to the variable incidence it has been reported that different types of digestive tract cancers may be influenced by lifestyle habits, such as physical exercise, smoking and diet (Rossi et al., 2006).

In 1987, Bird described aberrant crypt foci (ACF) as pre-neoplastic lesions in the colon and rectum of rats exposed to the chemical carcinogen 1,2-dimethylhydrazine (DMH) (Bird, 1987). DMH is able to stimulate cell division and induce colorectal ACF and tumor formation by interfering with DNA methylation, in a manner similar to that which occurs in humans (Poriet et al., 1993). The ACF manifested in the early stages of colorectal carcinogenesis as expansions of the intestinal crypts due to dysplasia, epithelial hyperplasia and hyperplasia (Magnuson et al., 1993; Bird, 1995). As determining the total number and size of ACF is simple, fast and inexpensive, DMH has been widely used in experimental models of colorectal cancer (Wargovich et al., 2000; Sequetto et al., 2013). Furthermore, the ACF evaluation is a short-term bioassay used to assess the role of chemopreventive agents in colorectal carcinogenesis (Wargovich et al., 2000; Newell and Heddle, 2004).

For decades, therapeutic approaches for the prevention and treatment of gastrointestinal cancers have been investigated and developed (Kanno et al., 2005; Dani et al., 2007; Araújo et al., 2011). Various biologically-active phytochemicals, especially flavonoids, have been investigated in relation to a possible
anti-carcinogenic effect (Moon et al., 2006; Pierini et al., 2008; Theodoratou et al., 2007). Epidemiological studies have suggested an association between flavonoid intake and a reduced incidence of inflammatory and cardiovascular disease and cancer (Jain and Parmar, 2011; Rossi et al., 2006). It was previously demonstrated that flavonoids, such as flavones, flavonols, catechins, procyanidins, flavanones, isoflavones, myricetin, naringenin, and hesperidin, have anti-carcinogenic potential in vitro (Pierini et al., 2008; Theodoratou et al., 2007; Moon et al., 2006). Furthermore, pre-clinical studies indicated that the anti-carcinogenic activity of flavonoids is related to the modulation of carcinogen metabolism, inflammation, and the regulation of cell proliferation and apoptosis, all of which are mechanisms that are directly implicated in the development and progression of colorectal cancers (Pierini et al., 2008; Talalay et al., 1988).

Currently, the significance of flavonoid dietary intake for the prevention and/or control of different types of cancer is highly controversial, particularly in humans, since the consumption of these phytochemicals is generally low and its metabolism extremely complex and not completely understood (Pierini et al., 2008; Spilsbury et al., 2012). Naringin is the main glycosylated flavonoid used in traditional Chinese medicine for its anti-inflammatory, antioxidant and anti-hypercholesterolemic activities. It is commonly found in citrus fruits like oranges, lemons, and grapefruit (Jain and Parmar, 2011; Liu et al., 2012). It has been reported that 1250 ml of orange and grapefruit juice contains approximately 690 mg of Naringin, representing the main natural source of this flavonoid (Scalbert and Williamson, 2000). After oral administration, Naringin and its secondary metabolites aglycone, naringenin, naringenin–glucuronide and naringenin–sulfate, were found in the urine and blood in rats (Liu et al., 2012). Furthermore, the unabsorbed fraction of the flavonoid is transformed by intestinal microflora in phenolic acids (Liu et al., 2012) that fall into several categories, such as simple phenols, phenolic acids, coumarins, flavonoids, hydrolysable and condensed tannins, lignans and lignins (Naczk and Shahidi, 2004). Recognizably, these substances are naturally occurring antioxidants, with a marked inhibitory effect on lipid, protein and nucleic acids oxidation both in vitro (Chen et al., 2010) and in vivo (Vanamala et al., 2006).

There is sufficient evidence that carcinogenesis may be influenced by phenol compounds (Master et al., 2012; Araújo et al., 2011). Although a possible anti-carcinogenic effect has been attributed to the flavonoid Naringin in different murine models of cancer (Magnuson et al., 1993; Kanno et al., 2005), the roles of this flavonoid in the treatment of pre-neoplastic lesions and prevention of colorectal cancer are still unknown. Thus, this study investigated the therapeutic effect of Naringin on pre-neoplastic lesions and colorectal pathological remodeling in a murine model of chemical carcinogenesis.

2. Materials and methods

2.1. Animals and experimental model

Female 8-week old Wistar rats weighing 130.8 ± 17.1 g were provided by the Central Animal Laboratory of the Federal University of Viçosa (Brazil). The animals were allocated individually in boxes with an automated ventilation system (Aleisco Ventilife®; São Paulo, Brazil) kept in an environment with regulated temperature (20 ± 2°C), humidity (60–70%) and light (12/12 h light/dark), receiving water and food ad libitum. The animals were divided into 5 groups with 6 animals in each group: Group 1 (control): 0.9% saline; Group 2 (control of induction): DMH + 0.9% saline; Group 3: DMH + Naringin (50 mg/kg); Group 4: DMH + Naringin (25 mg/kg); Group 5: DMH + Naringin (2.5 mg/kg). The doses of Naringin were determined in a context of dietary supplementation, considering 50%, 25% and 2.5% of the effective dose (400 mg/kg/day) able to reduce cardiovascular risk factors in humans (Jung et al., 2003) and oxidative stress in rats (Singh and Chopra, 2004). The study was conducted according to internationally accepted standards for the use and care of laboratory animals and approved by the Animal Ethics Committee of the institution (protocol approval 00002/2012-1).

Colorectal carcinogenesis was induced by one subcutaneous injection of 1.2-dimethylhydrazine (Aldrich Chemical Co., Milwaukee, USA) at 20 mg/kg every week for 10 weeks (Sequetto et al., 2013). From the 11th week, the flavonoid Naringin (purity > 95%; Aldrich Chemical Co., Milwaukee, USA) diluted in 700 μl of 0.9% saline (vehicle) was administered by gavage every 48 h for 14 weeks. The animals in G1 received only 0.9% saline (700 μl). The animals’ weight was recorded weekly. At the end of 25 weeks the animals were euthanized by cervical dislocation under anesthesia (ketamine 10 mg/kg and xylazine 2 mg/kg, i.p.) after 12 h of fasting. This protocol was previously tested by our research group and showed good reproducibility and efficiency to induce colorectal pre-neoplastic lesions with low frequency of tumors (Sequetto et al., 2013).

2.2. Screening of aberrant crypt foci

After euthanasia the large intestines were removed, divided into three equal pieces (proximal colon, middle colon, and distal colon + rectum) in relation to the cecum. The intestines were washed in 0.9% saline, longitudinally opened and fixed for 24 h in paraaffin plates containing 10% buffered formalin, pH = 7.2 (Bird, 1987). The intestinal pieces were stained with 0.1% methylene blue for 1 min and rinsed in phosphate buffer (pH = 7.2) for microscopic analysis. For ACF identification and quantification, the surface of the intestinal mucosa was observed from the intact pieces using a light microscope (Olympus BX-60®, Tokyo, Japan) with >10 objective lens (Bird, 1987, 1995). The entire intestine was evaluated and the ACF categorization was performed by determining the observed frequency of aberrant crypts for each focus. Thus, the established categories included foci with 1, 2, 3, 4–10, >10 aberrant crypts, and tumor (Sequetto et al., 2013).

2.3. Histopathology, mucin histochemistry and histomorphometry

After ACF analysis, the intestinal fragments were destained with successive washes in 5% ethanol, dehydrated in ethanol, diaphanized in xylene and embedded in paraaffin. 5 μm-thick sections were obtained using a rotary microtome (Leica Multicut 2045®, Reichert-Jung Products, Germany). The sections were stained with hematoxylin and eosin (H&E) for general morphological characterization, periodic acid-schiff (PAS) for neutral mucins, and Alcian blue (AB) for acidic mucins, (Yoshimine et al., 2004; Meira et al., 2008). To avoid the histological analysis of the same histological area in 20 tissue sections were used. Sections were viewed and images were captured using a light microscope (Olympus BX-60®, Tokyo, Japan) connected to a digital camera (Olympus QColor-3®, Tokyo, Japan). For each staining method and animal, ten microscopic fields were sampled with a >40 objective lens in each intestinal segment, so that a total area of 5.72 × 106 μm2 was analyzed for each group. In animals exposed to DMH, all histological fields analyzed showed normal and aberrant crypts. Areas of tumor tissue were not included in the count of mucous-secreting cells.

The histopathological parameters analyzed were the presence of dysplasia, crypt dilation and enterocyte morphology (Meira et al., 2008). From longitudinal sections stained with H&E, the crypt lengths and widths were determined. Cross-sectioned crypts were used to determine the area of each crypt and its lumen. The volume of each crypt and lumen was estimated by multiplying the crypt length by the areas previously measured. Sections stained with AB and PAS were used to determine the number of mucous-secreting cells per unit of histological area (Sequetto et al., 2013). In these sections, the volume density (Vv) occupied by acid (AB+) and neutral (PAS+) mucins was estimated by point counting using the following formula: Vv[AB+] + PAS+ = P[AB+] + PAS+/P, where P is the number of points that detect each mucin type and P is the total number of test points. For this analysis a test system with 72 points applied to a standard histological area of 20.0 × 103 μm2 was used (Mandarim-de-Lacerda, 2003). All histomorphometric analysis was performed using the software Image-Pro Plus 4.5® (Media Cybernetics, Silver Spring, MD, USA).

2.4. Cell division and AgNOR

Mitotic cells were identified in the crypts according Miyamoto et al. (2006). For each animal, ten microscopic fields stained with H&E were randomly sampled at ×400 magnification in each intestinal segment. In animals exposed to DMH, all histological fields analyzed showed normal and aberrant crypts. Areas of tumor tissue were not included in this analysis. The number of mitotic figures per histological area was determined on longitudinal sections that allowed evaluation of the whole crypt from the bottom to the base. Results were expressed for the whole intestine as the number of mitoses per m2.

From serial sections used in the analysis of mitotic cells, argyrophilic nucleolar organizing regions (AgNORs) were marked according to the method described by Howell and Black (1982). Briefly, 4-μm thick sections obtained from each intestinal segment were incubated in the dark with colloidal silver solution for 60 min at room temperature. AgNORs were visualized under a light microscope (Olympus BX-60®, Tokyo, Japan) at >1000 magnification and counted in 90 enterocyte nuclei in 30 crypts that were randomly sampled from each intestinal segment for each animal. Results were expressed for the whole intestine as the number of AgNORs per nucleus.
2.5. Scanning electron microscopy and mineral microanalysis

Fragments of the three intestinal segments (proximal, middle and distal + rectum) were dissected, dehydrated in ethanol and dried at the critical point (CPD 030®, Bal-Tec, Witten, North Rhine-Westphalia, Germany). The mineral distribution and contents were investigated by X-ray Energy Dispersive Spectroscopy (EDS) using a scanning electron microscope (Leo 1430VP, Zeiss, Jena, Germany) coupled to an X-ray detector (Tracer TN5502, Middleton, WI, USA). For this analysis a 300× magnification, a 20 kV acceleration voltage and a 19 mm working distance was applied. For each group a total area of 36.18 × 10^2 μm^2 was investigated in each intestinal segment. The proportions of the elements carbon (C), nitrogen (N), oxygen (O), fluorine (F), sodium (Na), magnesium (Mg), phosphorus (P), sulfur (S), chlorine (Cl), potassium (K), calcium (Ca), manganese (Mn), iron (Fe), copper (Cu), selenium (Se) and zinc (Zn) were measured and expressed as the average value for the whole intestine (Sequetto et al., 2013).

2.6. Hematological and biochemical analysis

Blood samples were collected by cardiac puncture at the time of euthanasia. An aliquot of total blood (2 ml) was collected in heparin-containing sterile disposable syringes and submitted to hematological analysis using an automated cell analyzer (Humacount Plus®, Human do Brazil, São Paulo, SP, Brazil). The remaining blood was centrifuged and the serum used for the biochemical determination of albumin, total protein, glucose, total cholesterol and triglycerides (Human in Vitro Diagnostics, Minas Gerais, Brazil) (Sequetto et al., 2013).

2.7. Genotoxicity assay

DNA oxidative damage was investigated after nucleic acid extraction and purification according to Coombs et al. (1999). Briefly, fragments of the colonic tissue (20 mg) removed before histological fixation were incubated with proteinase K (10 mg/ml) at 55°C. Genomic DNA was extracted by the phenol–chloroform (1:1) method. From isolated DNA, tissue levels of 8-hydroxy-2’-deoxyguanosine (8-OHdG) were determined according to Kakimoto et al. (2002). Briefly, DNA was resuspended in 10 mmol/l Tris–HCl and 0.1 mmol/l EDTA. Five microliters of 200 mmol/l sodium acetate buffer and 5 μg nuclelease P1 (Aldrich Chemical Co., Milwaukee, USA) were added to 45 μl DNA samples. The mixtures were incubated at 37°C for 1 h to digest the DNA to nucleotides. Then, 5 μl of 500 mmol/l Tris–HCl, 10 mmol/l MgCl2 and 0.6 units alkaline phosphatase (Aldrich Chemical Co., Milwaukee, USA) were added to the samples. The mixtures were incubated at 37°C for 1 h to hydrolyze the nucleotides to nucleosides. The nucleoside samples were used for the determination of 8-OHdG by competitive enzyme-linked immunosorbent assay (Cell Biolabs Inc., San Diego, CA, USA).

2.8. Statistical procedure

Results were expressed as mean and standard deviation (mean ± SD). The normality of the data distribution was verified using the D’agostino–Pearson test (1:1 method). From isolated DNA, tissue levels of 8-hydroxy-2’-deoxyguanosine (8-OHdG) were determined according to Kakimoto et al. (2002). Briefly, DNA was resuspended in 10 mmol/l Tris–HCl and 0.1 mmol/l EDTA. Five microliters of 200 mmol/l sodium acetate buffer and 5 μg nuclelease P1 (Aldrich Chemical Co., Milwaukee, USA) were added to 45 μl DNA samples. The mixtures were incubated at 37°C for 1 h to digest the DNA to nucleotides. Then, 5 μl of 500 mmol/l Tris–HCl, 10 mmol/l MgCl2 and 0.6 units alkaline phosphatase (Aldrich Chemical Co., Milwaukee, USA) were added to the samples. The mixtures were incubated at 37°C for 1 h to hydrolyze the nucleotides to nucleosides. The nucleoside samples were used for the determination of 8-OHdG by competitive enzyme-linked immunosorbent assay (Cell Biolabs Inc., San Diego, CA, USA).

3. Results

3.1. Biometry and aberrant crypt foci

The increase in body weight was similar throughout the experiment. There was no significant difference in body weight (Fig. 1) and intestinal length (data not shown) between the groups at the beginning (11th week) and at the end (25th week) of the flavonoid treatment.

The analysis of the intestinal mucosal surface showed the presence of ACF in all intestinal segments in the groups exposed to DMH. The ACF were distributed throughout the entire intestinal surface. Different ACF development levels were observed, with a predominance of foci containing between 1 and 3 aberrant crypts surrounded by normal crypts, distributed mainly in the middle and distal colon (Fig. 2).

The quantification of mucosal lesions showed an increased number of ACF in all intestinal segments in the groups exposed to DMH, especially G2. In general, it was observed that all doses of Naringin significantly reduced the total number of aberrant crypts per focus in relation to G2. For all intestinal segments, the best results were observed in G5. For all groups treated with the flavonoid, the ACF reduction was higher in the middle and proximal colon. There was low frequency of tumors in animals exposed to DMH. The treatments with the flavonoid were not always efficient in reducing the number of these tumors (Table 1).

3.2. Histopathology, histochemistry and histomorphometry

Histopathological analysis indicated a marked morphological reorganization of intestinal crypts. In G2 and G3 hypertrophic crypts with severe dysplasia were observed, mainly in the basal third. In these groups, there was hypertrophy and an increased density of enterocytes with elongated nuclei, a marked reduction in mucus-producing cell density throughout the crypt length, and a dilatation of the luminal space. In G4 and G5, there was an evident reduction of the crypt dysplasia (Fig. 3).

The analysis of the crypts showed a significant increase in all histomorphometric parameters evaluated in G2 compared to G1 for all intestinal segments. In general, all of the treated groups showed a reduction of these parameters compared to G2, mainly in the proximal colon. Regardless of the intestinal segment analyzed, G3 showed no statistical differences in any histomorphometric parameter compared to G2. These parameters were significantly lower for all intestinal segments in G5 compared to G2 (Table 2).

The histochemical analysis indicated a significant reduction in acidic and neutral mucin expression and the density of mucous-secreting cells in the crypts for all intestinal segments in G2 and G3 compared to G1. In G4 and G5, the expression of mucins and the density of goblet cells were significantly higher compared to G2. In G5, the number of mucous-secreting cells was similar, while the proportion of the histological area occupied by mucins was significantly lower than in G1 (Fig. 4 and Table 3).

For all intestinal segments, there was significant increase in number of nucleolar organizer regions per nucleus of enterocytes (AgNORs/nucleus) and mitosis in G2 and G3 compared to G1. In G4 and G5, the number of AgNORs/nucleus and mitosis was significantly lower compared to G2. There was no statistical difference in these parameters between G1 and G5 (Fig. 5).

3.3. Biochemistry and minerals

The EDS analysis indicated a homogeneous mineral distribution in the mucosal for all intestinal segments both on the ACF dysplastic epithelium (Fig. 6) and on the normal epithelium (data not shown). The mineral quantification indicated a significant reduction in the tissue levels of Mg, Mn, Cu, Se and Zn, and a significant increase in Cl, Fe, Na and K in G2 and G3 compared to G1. In G4 and G5, the levels of Mg, Mn, Cu, Se, Na and K were significantly higher, while the levels of Cl, Fe, Na, and K were significantly lower in G5 compared to G1 (Table 3).
lower than G2, with the best results found in G5, which was similar to G1 (Fig. 6).

There were no significant differences in the biochemical parameters between the groups, except for total protein, which was significantly reduced in G2 (18.7%) compared to G1, and increased in G5 (16.8%) compared to G2 (Table 4). The hematological parameters were similar in all groups, except for the number of red blood cells. This parameter was significantly reduced in G2 and G3 compared to G1 and G5, which were similar between them (Table 5).

The levels of 8-OHdG in the genomic DNA extracted from colonic tissue were significantly increased in G2, G3 and G4 compared to G1. This parameter was significantly reduced in G4 and G5 compared to G2. Similar results in G1 and G5 were observed (Fig. 7).

4. Discussion

In the present study, the effect of the flavonoid Naringin was investigated on colorectal pre-neoplastic lesions induced by chemical carcinogen. The results showed that the flavonoid partially inhibited the morphological reorganization of the intestinal epithelium, reducing the ACF number, the total number of aberrant crypts per focus, the number of AgNORs/nucleus, the intensity of the crypt epithelial dysplasia and increasing the mucus expression and the density of mucus-producing cells.

The ACF reduction in treated rats was greater in the middle and proximal colon, characterizing the sensitivity of these segments to the flavonoid. In addition to the systemic effects, there is evidence...
indicating a local action of flavonoids in the intestinal mucosa, especially in the initial segments. It is suggested that this event is related to the progressive degradation and inactivation of the flavonoids and its bioactive-derived compounds along the intestinal tract with a higher degradation rate in the distal segments of the intestine (Sequetto et al., 2013; Wolffram et al., 2002). A greater susceptibility of the distal colonic mucosa to DMH should also be considered. This feature can be associated with a higher rate of cell turnover, determined by the microenvironment that exposes the distal mucosa to contact with high levels of toxins derived from food digestion and bacterial metabolism, as well as physically-abrasive forces associated with the transport and storage of a more consistent fecal material (Choudhary and Hansen, 1998; Ullman and Itzkowitz, 2011).

In all animals exposed to DMH a low tumor frequency was observed, an event that is related to the experimental protocol applied, which was designed to minimize the development of advanced lesions (Sequetto et al., 2013). In previous investigations, the distal mucosa to contact with high levels of toxins derived from food digestion and bacterial metabolism, as well as physically-abrasive forces associated with the transport and storage of a more consistent fecal material (Choudhary and Hansen, 1998; Ullman and Itzkowitz, 2011).

Fig. 3. Representative photomicrographs of longitudinally sectioned crypts from the distal colon of rats with chemically-induced colorectal pre-neoplastic lesions treated with the flavonoid Naringin. Normal crypts with narrow lumen and high density of goblet cells are seen in G1. In G2 and G3, crypts with severe dysplasia and elongated enterocytes are observed, particularly at the crypt base, as well as a low density of goblet cells. In G4 crypts with slightly dilated bases and elongated enterocytes are observed. The crypts in G5 are similar to G1 (H&E staining, bars = 50 μm). Arrows = areas of dysplasia. G1: 0.9% saline, G2: DMH, G3: DMH + Naringin (10 mg/kg), G4: DMH + Naringin (100 mg/kg), G5: DMH + Naringin (200 mg/kg).

Table 2
Morphometric parameters of colonic crypts in rats exposed to chemical carcinogen and treated with the flavonoid Naringin.

<table>
<thead>
<tr>
<th>Intestinal segment</th>
<th>Length (μm)</th>
<th>Width (μm)</th>
<th>Cript area (μm²)</th>
<th>Luminal area (μm²)</th>
<th>Cript volume (μm³) x 10⁻³</th>
<th>Luminal volume (μm³) x 10⁻³</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>215.1 ± 30.4</td>
<td>36.9 ± 6.2</td>
<td>1649.3 ± 387.2</td>
<td>152.7 ± 43.9</td>
<td>404.2 ± 75.9</td>
<td>32.9 ± 9.5</td>
</tr>
<tr>
<td>G2</td>
<td>330.9 ± 36.7</td>
<td>72.1 ± 15.7</td>
<td>3005.1 ± 471.1</td>
<td>471.3 ± 59.5</td>
<td>994.4 ± 70.1</td>
<td>156.0 ± 25.7</td>
</tr>
<tr>
<td>G3</td>
<td>262.0 ± 45.3</td>
<td>44.0 ± 10.7</td>
<td>2518.4 ± 397.7</td>
<td>332.8 ± 67.7</td>
<td>567.7 ± 66.4</td>
<td>83.3 ± 11.3</td>
</tr>
<tr>
<td>G4</td>
<td>268.3 ± 17.8</td>
<td>38.4 ± 7.0</td>
<td>1982.0 ± 429.1</td>
<td>145.1 ± 51.6</td>
<td>453.2 ± 85.3</td>
<td>38.9 ± 9.4</td>
</tr>
<tr>
<td>G5</td>
<td>241.6 ± 40.5</td>
<td>39.6 ± 7.6</td>
<td>2110.7 ± 466.3</td>
<td>151.5 ± 50.2</td>
<td>422.9 ± 76.1</td>
<td>36.6 ± 8.6</td>
</tr>
<tr>
<td>Medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>243.3 ± 27.9</td>
<td>35.7 ± 7.9</td>
<td>1685.4 ± 415.2</td>
<td>170.4 ± 68.2</td>
<td>410.1 ± 65.9</td>
<td>41.5 ± 9.3</td>
</tr>
<tr>
<td>G2</td>
<td>390.1 ± 55.0</td>
<td>65.2 ± 9.8</td>
<td>3100.3 ± 698.1</td>
<td>519.3 ± 118.8</td>
<td>1209.4 ± 100.7</td>
<td>202.6 ± 39.5</td>
</tr>
<tr>
<td>G3</td>
<td>300.2 ± 50.9</td>
<td>55.4 ± 6.1</td>
<td>2813.9 ± 518.4</td>
<td>348.3 ± 100.1</td>
<td>722.7 ± 103.0</td>
<td>87.9 ± 16.4</td>
</tr>
<tr>
<td>G4</td>
<td>270.7 ± 35.9</td>
<td>52.1 ± 7.2</td>
<td>1940.9 ± 443.2</td>
<td>323.0 ± 67.2</td>
<td>596.2 ± 85.8</td>
<td>64.4 ± 9.3</td>
</tr>
<tr>
<td>G5</td>
<td>250.4 ± 33.1</td>
<td>45.2 ± 7.0</td>
<td>1817.5 ± 494.7</td>
<td>228.2 ± 88.7</td>
<td>562.7 ± 90.0</td>
<td>45.9 ± 8.1</td>
</tr>
<tr>
<td>Distal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>266.5 ± 30.7</td>
<td>33.7 ± 6.0</td>
<td>1531.1 ± 425.4</td>
<td>114.2 ± 55.7</td>
<td>408.0 ± 79.5</td>
<td>30.5 ± 8.0</td>
</tr>
<tr>
<td>G2</td>
<td>392.0 ± 50.5</td>
<td>68.1 ± 10.5</td>
<td>2832.6 ± 437.8</td>
<td>325.0 ± 85.3</td>
<td>1110.4 ± 112.5</td>
<td>127.4 ± 26.9</td>
</tr>
<tr>
<td>G3</td>
<td>342.8 ± 31.4</td>
<td>60.0 ± 8.6</td>
<td>2550.4 ± 508.2</td>
<td>286.7 ± 67.1</td>
<td>712.1 ± 91.7</td>
<td>64.8 ± 10.7</td>
</tr>
<tr>
<td>G4</td>
<td>314.5 ± 47.3</td>
<td>52.8 ± 6.9</td>
<td>2031.0 ± 599.2</td>
<td>235.2 ± 52.8</td>
<td>669.5 ± 106.5</td>
<td>53.2 ± 10.2</td>
</tr>
<tr>
<td>G5</td>
<td>240.2 ± 40.5</td>
<td>42.3 ± 9.1</td>
<td>1678.7 ± 459.6</td>
<td>114.0 ± 52.9</td>
<td>451.2 ± 82.9</td>
<td>47.3 ± 9.5</td>
</tr>
</tbody>
</table>

G1: 0.9% saline, G2: DMH, G3: DMH + Naringin (10 mg/kg), G4: DMH + Naringin (100 mg/kg), G5: DMH + Naringin (200 mg/kg). Values are expressed as mean and standard deviation (mean ± SD). Statistical difference for the same intestinal segment p < 0.05, vs. G1, vs. G2.
a higher incidence of tumors was achieved from 28 weeks of DMH exposure (Bird, 1995; Roberts et al., 2002). However, the experimental model used in this study was designed considering a therapeutic approach in an attempt to accelerates the regression and/or inhibit the progression and severity of pre-established pre-neoplastic lesions. A chemopreventive approach can also be considered in order to preventing the development of colorectal cancer from pre-neoplastic lesions in evolution. Although treatment with the flavonoid has been effective in reducing the ACF, a similar effect was not observed in advanced lesions (tumor). Thus, it is suggested that Naringin may potentially have an inhibitory activity in the early stages of carcinogenesis, with a limited effect on tumor lesions that were previously established. Similar findings were observed in a previous study conducted by our research group with the flavonoid chrysin (Sequetto et al., 2013). This aspect is poorly understood, however, it is possible that some molecular routes of cell proliferation and differentiation modulated by phenol compounds in normal cells become up-regulated and unresponsive in tumor cells (Lee et al., 2005; Ramos et al., 2011), a hypothesis that requires confirmation.

The results indicated a possible dose-dependent inhibitory effect of the flavonoid Naringin on pre-neoplastic lesions in all the intestinal segments. The best results were found in the group receiving the highest dose of the flavonoid (200 mg/kg).
Fig. 5. Argyrophilic nucleolar organizer regions (AgNORs) and mitosis in the colonic epithelium of rats with chemically-induced colorectal pre-neoplastic lesions treated with the flavonoid Naringin. (A) A representative photomicrograph of the control group (G1) indicating enterocytes with ovoid nuclei and one or two AgNORs (bar = 15 μm). (B) Note the elongated nuclei with three (arrowheads) to seven (arrow) AgNORs in animals exposed to DMH (G2) (bar = 15 μm). (C) Number of AgNORs per nuclei for all groups. (D and E) Mitotic figures in the colonic cripts (bar = 10 μm). (F) Number of mitosis in the colonic epithelium for all groups. G1: 0.9% saline, G2: DMH, G3: DMH + Naringin (10 mg/kg), G4: DMH + Naringin (100 mg/kg), G5: DMH + Naringin (200 mg/kg). Values are expressed as mean and standard deviation (mean ± SD) for the whole intestines. Statistical difference *p < 0.05, †vs. G1, ‡vs. G2.

Fig. 6. Elemental distribution in the colonic epithelium of rats with chemically-induced colorectal pre-neoplastic lesions treated with the flavonoid Naringin. The images represent the distribution map of the different elements in a focus with two aberrant crypts of an animal in the Group 2. Note the homogeneity in elemental distribution. The table indicates the concentration of elements with significant differences among the groups. G1: 0.9% saline, G2: DMH, G3: DMH + Naringin (10 mg/kg), G4: DMH + Naringin (100 mg/kg), G5: DMH + Naringin (200 mg/kg). Values are expressed as mean and standard deviation (mean ± SD) for the whole intestine. Statistical difference *p < 0.05, †vs. G1, ‡vs. G2.
Hematological parameters of rats with chemically-induced colorectal pre-neoplastic lesions treated with the flavonoid Naringin.

Serum biochemical parameters of rats with chemically-induced colorectal pre-neoplastic lesions treated with the flavonoid Naringin.

be due to an inhibitory effect of the Naringin on cell proliferation. It is well established that the reduction in the number of pre-neoplastic lesions may be ascribed to oxidative DNA damage (Tanaka et al., 1997). Thus, the decreased number of AgNORs/nucleus suggests an inhibition of DNA synthesis in the cells of animals exposed to chemical carcinogens (Tanaka et al., 1997). The correlation between the high rate of cell proliferation and an increased number of AgNORs/nucleus in different cancers has been described by Jain and Parmar (2011), who found a significant reduction of the lipid peroxidation and activities of superoxide dismutase, catalase and glutathione reductase from colonic tissue of rats with chemically-induced colorectal pre-neoplastic lesions treated with the flavonoid. These findings indicated that Naringin demonstrated both in vitro (Araújo et al., 2011; Ramos et al., 2011) and in vivo (Lee et al., 2005; Tanaka et al., 1997). The inhibitory effect of flavonoids against gastric cancer cells was demonstrated by Lee et al. (2005). Fukai et al. (2002) reported that flavonoids from Glycyrrhiza glabra may be useful as chemopreventive agents for peptic ulcers or gastric cancer in individuals infected with Helicobacter pylori. In addition, Kajimoto et al. (2002) verified the action of flavonoids in the inhibition of cell growth and the induction of apoptosis in various cancer cell lines, such as MKN7 cells from human gastric cancers. Lee et al. (2005) suggested that the anti-tumor effects of Naringenin are mediated by its capacity to down-regulate the cell signaling mediated by β-catenin/Tcf, an important route that is related to the initial stages of gastric carcinogenesis. Vanamala et al. (2006) reported a chemopreventive effect of dietary supplementation with flavonoids isolated from grapefruit, including Naringin (200 mg/kg) on colon carcinogenesis induced by azoxymethane. In a study conducted by Seo et al. (2003), the addition of up to 1250 mg/kg of Naringin in the diet was well tolerated in Sprague–Dawley rats. Thus, it is believed that an intake of this flavonoid below 1250 mg/kg is potentially safe in murine models, minimizing the possibility of damage to organs and healthy tissues. These findings are corroborated by the results of animal weights, biochemical and hematological parameters, which indicated no systemic toxicity of this flavonoid in the doses used.

The high levels of 8-OHdG in colonic tissue of animals exposed to DMH indicated oxidative damage to genomic DNA, which was attenuated by Naringin treatment in a dose-dependent way. The anti-inflammatory effects of Naringenin were verified by Jain and Parmar (2011), who found a significant reduction of the lipid peroxidation and activities of superoxide dismutase, catalase and glutathione reductase in the plasma and tissues of rats treated with the flavonoid. These findings indicated that Naringin

---

**Table 4**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total protein (g/dL)</td>
<td>84.2 ± 4.1</td>
<td>84.1 ± 3.9</td>
<td>84.2 ± 3.9</td>
<td>84.1 ± 3.8</td>
<td>84.2 ± 3.8</td>
</tr>
<tr>
<td>Albumin (g/dL)</td>
<td>3.8 ± 0.4</td>
<td>3.8 ± 0.4</td>
<td>3.8 ± 0.4</td>
<td>3.8 ± 0.4</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>183.7 ± 20.1</td>
<td>183.7 ± 20.1</td>
<td>183.7 ± 20.1</td>
<td>183.7 ± 20.1</td>
<td>183.7 ± 20.1</td>
</tr>
<tr>
<td>Cholesterol (mg/dL)</td>
<td>61.9 ± 13.7</td>
<td>61.9 ± 13.7</td>
<td>61.9 ± 13.7</td>
<td>61.9 ± 13.7</td>
<td>61.9 ± 13.7</td>
</tr>
<tr>
<td>Glucose (mg/dL)</td>
<td>112.7 ± 28.1</td>
<td>112.7 ± 28.1</td>
<td>112.7 ± 28.1</td>
<td>112.7 ± 28.1</td>
<td>112.7 ± 28.1</td>
</tr>
</tbody>
</table>

---

**Table 5**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>G1</th>
<th>G2</th>
<th>G3</th>
<th>G4</th>
<th>G5</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBC (10⁹/µL)</td>
<td>6.8 ± 1.3</td>
<td>6.8 ± 1.3</td>
<td>6.8 ± 1.3</td>
<td>6.8 ± 1.3</td>
<td>6.8 ± 1.3</td>
</tr>
<tr>
<td>RBC (10⁶/µL)</td>
<td>7.5 ± 1.8</td>
<td>7.5 ± 1.8</td>
<td>7.5 ± 1.8</td>
<td>7.5 ± 1.8</td>
<td>7.5 ± 1.8</td>
</tr>
<tr>
<td>PLT/C10⁹/µL</td>
<td>20.0 ± 6.5</td>
<td>20.0 ± 6.5</td>
<td>20.0 ± 6.5</td>
<td>20.0 ± 6.5</td>
<td>20.0 ± 6.5</td>
</tr>
<tr>
<td>HGB (g/dL)</td>
<td>8.5 ± 0.4</td>
<td>8.5 ± 0.4</td>
<td>8.5 ± 0.4</td>
<td>8.5 ± 0.4</td>
<td>8.5 ± 0.4</td>
</tr>
<tr>
<td>HCT (%)</td>
<td>15.4 ± 0.4</td>
<td>15.4 ± 0.4</td>
<td>15.4 ± 0.4</td>
<td>15.4 ± 0.4</td>
<td>15.4 ± 0.4</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>43.8 ± 1.9</td>
<td>43.8 ± 1.9</td>
<td>43.8 ± 1.9</td>
<td>43.8 ± 1.9</td>
<td>43.8 ± 1.9</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>54.3 ± 2.3</td>
<td>54.3 ± 2.3</td>
<td>54.3 ± 2.3</td>
<td>54.3 ± 2.3</td>
<td>54.3 ± 2.3</td>
</tr>
<tr>
<td>MCHC (g/dL)</td>
<td>19.1 ± 1.0</td>
<td>19.1 ± 1.0</td>
<td>19.1 ± 1.0</td>
<td>19.1 ± 1.0</td>
<td>19.1 ± 1.0</td>
</tr>
<tr>
<td>RDW (%)</td>
<td>35.2 ± 1.0</td>
<td>35.2 ± 1.0</td>
<td>35.2 ± 1.0</td>
<td>35.2 ± 1.0</td>
<td>35.2 ± 1.0</td>
</tr>
<tr>
<td>PLT (%)</td>
<td>15.5 ± 1.0</td>
<td>15.5 ± 1.0</td>
<td>15.5 ± 1.0</td>
<td>15.5 ± 1.0</td>
<td>15.5 ± 1.0</td>
</tr>
<tr>
<td>MPV (fL)</td>
<td>8.5 ± 0.4</td>
<td>8.5 ± 0.4</td>
<td>8.5 ± 0.4</td>
<td>8.5 ± 0.4</td>
<td>8.5 ± 0.4</td>
</tr>
</tbody>
</table>

---

**Fig. 7.** Levels of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in DNA samples extracted from colonic tissue of rats with chemically-induced colorectal pre-neoplastic lesions treated with the flavonoid Naringin. G1: 0.9% saline, G2: DMH, G3: DMH + Naringin (10 mg/kg), G4: DMH + Naringin (100 mg/kg), G5: DMH + Naringin (200 mg/kg). The values are expressed as mean and standard deviation (mean ± SD). Statistical difference \( p < 0.05 \), ‘vs. G1’, ‘vs. G2’.
has an intrinsic antioxidant activity, which is partly independent of its effects on the expression and/or activity of cellular antioxidant enzymes. Moreover, the administration of Naringin was previously associated with a decreased synthesis of NF-kappa-β (nuclear factor kappa-β) and activity of nitric oxide synthase, which are components involved in cellular pro-oxidant events during carcinogenesis (Kanno et al., 2006).

Several minerals in the diet stand out as important protective or modulating agents for the risk against the development of malignancies (Park et al., 2011). Minerals such as copper, magnesium, selenium, and zinc play an important role in the stages of initiation, promotion and cancer progression (Jaiswal and Narayanan, 2004; Dani et al., 2007). In this study, these minerals were markedly reduced in the ACF dysplastic epithelium in all intestinal segments. According to Anetor et al. (2007), micronutrients such as zinc may reduce the risk of cancer due to its antioxidant properties, since pro-oxidants events are involved in various stages of carcinogenesis. It has been reported that this mineral is capable of inhibiting the growth and proliferation of many types of tumor cells, including colon cancer cells (Park et al., 2002; Klein et al., 2006). Studies with animal models and cancer cell cultures indicated that selenium and selenoproteins are involved in the maintenance of bowel function and the prevention of colorectal cancer (Méplan and Hesketh, 2012; Duffield-Lillico et al., 2002). Low levels of antioxidant enzymes that are dependent on selenium and zinc, which exhibit important antagonistic action in carcinogenesis, have been demonstrated in cells and cancerous tissues, (Grigolo et al., 1998). According Méplan and Hesketh (2012), although there is no strong epidemiological evidence to link the cancer risk to serum and tissue Se levels, experimental evidence suggests an important role of Se and selenoproteins in the function of epithelial cells of the colonic mucosa, which is associated with the inhibition of the initiation and progression of premalignant lesions. An aspect that should also be emphasized is the elevation of tissue iron levels. It has been reported that the increase in tissue iron stores may promote the growth of cancer cells, which is potentially related to the catalytic activity of iron on the formation of hydroxyl radicals (Fenton reaction), with consequent oxidative damage to lipids, proteins and DNA, an inhibition of the cell’s immune defense, and a favoring of cell hyperplasia (Okada, 1996; Weinberg, 1996). Although the quantification of iron has been indicated as being potentially useful in the prognosis of patients with cancer (Weinberg, 1996), the current evidence associating iron and colon cancer remain extremely limited and controversial, requiring further investigation.

The results indicated that the flavonoid Naringin, especially at 200 mg/kg, was effective in minimizing pathological colorectal remodeling, thereby reducing the number of pre-neoplastic lesions in rats exposed to the chemical carcinogen DMH. The initial and medium colonic segments appear to be more sensitive to the inhibitory effect of this flavonoid on the crypt dysplasia, an effect that apparently has a limited influence on pre-established tumors. It is believed that the results may be due to the effects of Naringin on the inhibition of cellular proliferation, reduction of tissue levels of iron, recovery of antioxidant mineral levels, and attenuation of DNA oxidation. Although these effects are possible, as metabolic oxidative markers and antioxidant enzymes were not completely evaluated, it was not possible to determine the relationship between the level of minerals and tissue oxidative status, which constitutes the main limitation of the study and indicates the need for future investigations.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

The authors thank the “Núcleo de Microscopia e Microanalise” of the Federal University of Viçosa (UFV) by the assistance in the X-ray Energy Dispersive Spectroscopy.

References


