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

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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.

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 (Pories et al., 1993). The ACF manifested in the early stages of colorectal carcinogenesis as expansions of the intestinal crypts due to dysplasia, epithelial hypertrophy 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

Abbreviations: ACF, aberrant crypt foci; AB, Alcian blue; AgNORs, argyrophilic nucleolar organizing regions; DMH, 1,2-dimethylhydrazine; EDS, X-ray energy dispersive spectroscopy; PAS, periodic acid-schiff; Vv, volume density; 8-OHdG, 8-hydroxy-2′-deoxyguanosine.

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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 (Alesco 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 (10 mg/kg); Group 4: DMH + Naringin (100 mg/kg); Group 5: DMH + Naringin (200 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/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-I).

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 paraffin 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 a ×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 50% ethanol, dehydrated in ethanol, diaphanized in xylene and embedded in paraffin. 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, (Yoshimi et al., 2004; Meira et al., 2008). To avoid the histological analysis of the same histological area 1 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 57.2×10^5 µm^2 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 mucus-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 mucus-secreting cells per unit of histological area (Sequetto et al., 2013). In these sections, the volume density (Vv) occupied by acidic (AB+) and neutral (PAS+) mucins was estimated by point counting using the following formula: $Vv[AB+; PAS+] = P_P[AB+; PAS+]/P_T$, where P_P is the number of points that detect each mucin type and P_T is the total number of test points. For this analysis a test system with 72 points applied to a standard histological area of 20.0×10^3 µm^2 was used (Mandarim-de-Lacerda, 2003). All histomorphometric analysis was performed using the software Image Pro-Plus 4.5® (Media Cybernetcs, 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 top to the base. Results were expressed for the whole intestine as the number of mitosis per mm^2.

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.