Effects of dual modified resistant indica rice starch on azoxymethane-induced incipient colon cancer in mice

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Abstract. In this study, the effects of different doses of dual modification-treated (DMT) indica rice resistant starch (IR-RS) on azoxymethane (AOM)-induced early colon cancer in mice were investigated. The investigated factors included body weight, gastrointestinal emptying rate, the number and morphology of aberrant crypt foci (ACFs) and the specific expressions of adenomatous polyposis coli (APC), B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and cytochrome *c* genes. The results demonstrated that DMT IR-RS controlled the increase in the body weights of the mice, increased the gastrointestinal emptying rates and reduced the numbers of ACFs and aberrant crypts. Reverse transcription-polymerase chain reaction revealed that DMT IR-RS promoted the expression of APC, Bax and cytochrome *c* and inhibited the expression of Bcl-2. These results demonstrate that a DMT IR-RS diet may induce apoptosis and has beneficial health effects in AOM-induced early colon cancer in mice.

Introduction

Colon cancer is the third most common cancer among women (after breast and lung cancers) and men (following prostate and lung cancers) and the third most common cancer-related cause of mortality globally, particularly in western and developed nations (1,2). In addition, the incidence of colon cancer is higher in men than women and strongly increases with age (3). At present, colon cancer is one of the most frequently encountered malignant tumors, and the incidence of colon cancer ranks third among global gastrointestinal tumors (4). Economic development and improvements in living standards have caused marked changes in people's daily diets, and the morbidity and mortality of colon cancer have exhibited concurrent rapid increases in the last two decades. Morbidity and mortality are significantly higher in developed coastal areas than in the mainland. The incidence of colon cancer has strongly increased globally and is closely associated with elements of the so-called western lifestyle (5).

Colon cancer has long been associated with apoptosis and polygenic mutations (4,6). The genetics of colon cancer have been actively researched in the final decades of the past century. The major genes identified as being associated with colon cancer are adenomatous polyposis coli (APC), B-cell lymphoma 2 (Bcl-2), Bcl-2-associated X protein (Bax) and cytochrome *c* (7). Studies have shown that APC is a tumor suppressor gene. APC gene mutations can be detected in nearly all familial large adenomas and in 60-80% of sporadic colorectal cancers (CRCs) (8,9). Apoptosis is regulated in part by the Bcl-2 gene, which promotes cell survival and lengthens cell life by hindering programmed cell death (1,10). The expression of the Bcl-2 gene in colon cancer is higher than that in normal colonic mucosa and gradually increases as adenomas progress to early adenocarcinomas (11). Additionally, Bcl-2 can prevent the release of cytochrome *c* from the mitochondria to the cytoplasm and thus inhibit apoptosis (12). Bax is a member of the Bcl-2 gene family whose products are associated with the Bcl-2-homologous proteins (13). The biological activities of Bcl-2 are antagonized by Bax. The main function of Bax is to accelerate cell apoptosis (14). Cytochrome *c* is a water-soluble protein that is encoded by nuclear genes with a molecular weight of 12-13 kDa. The main function of cytochrome *c* is to adjust mitochondrial energy metabolism (15). Studies have revealed that cytochrome *c* also plays an important role in cell apoptosis via the transmission of the apoptosis signal and amplification of the regulation of apoptosis (15,16).

The occurrence of colon cancer is attributable to a number of causes, but epidemiological studies have shown that dietary factors are important in the prevention of human colon cancer (17). Understanding the cause of colon cancer would undoubtedly contribute to better surveillance and early

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Abbreviations: IR-RS, indica rice resistant starch; DMT, dual modification-treated; LG, low-dose group; MG, middle-dose group; HG, high-dose group; PC, positive control; MC, model control; NC, normal control; DW, distilled water; AOM, azoxymethane; CRC, colorectal cancer; ACF, aberrant crypt foci; RT-PCR, reverse transcription-polymerase chain reaction

Key words: resistant starch, gastrointestinal emptying rates, aberrant crypt foci, aberrant crypts, colon cancer

prevention and thus reduce cancer morbidity (18). Increasing the amount of fiber in the diet should reduce the incidence of cancers, particularly those of the colon and rectum (19,20). Furthermore, resistant starch (RS) can reduce the incidence of colon cancer (21). Therefore, improved eating habits and greater dietary adjustments are the most economical and effective means of prevention and control.

RS is a starch, the chemical structure of which is different from that of fiber, and the properties of RS are similar to those of soluble fiber. RS is a new food ingredient and has a low glycemic index. RS is termed an anti-digested starch on the basis of the fraction of the starch that cannot be digested in the small intestine and is instead partially fermented in the large intestine to produce short-chain fatty acids and other products (22). In our previous study, RS was prepared from indica starch using a new method that combines α-amylase, pullulanase and heat-moisture treatment. Indica rice resistant starch (IR-RS) products produce a mixture of B- and V-type X-ray diffraction patterns and a crystallinity of 51.0% (23). *In vivo* experiments revealed that IR-RS is able to improve the symptoms associated with high blood sugar and the complications of diabetes in mice. Building upon our previous study, this study primarily focuses on the effect of IR-RS on azoxymethane (AOM)-induced colon cancer in mice and illuminates the mechanism of action of this effect. The results of this study should provide a scientific basis for colon cancer prevention and control measures, and be highly significant for the prevention of chronic diseases.

Materials and methods

Materials. Dual modification-treated (DMT) IR-RS was prepared according to the procedure outlined by Zhou *et al* (23). The RS content of the DMT IR-RS was 51.3%. AOM was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methylene blue, 10% buffered formalin and other chemicals and reagents were analytical grade.

Animals. One hundred male SCXK (su) 2011-001 mice (SPF mice that are free of specific microbes and parasites and so reduce experimental interference) were purchased from Changzhou Card Vince Laboratory Animal Co., Ltd. (`Changzhou, China). The experiments were approved by the ethical committee of the Animal Experiment Committee of China and conform to national guidelines on the care and use of laboratory animals (24).

AOM-induced colon cancer mice. During the experiments, the mice were housed in an approved laboratory animal facility and maintained under controlled temperature (25˚C) and lighting (12 h light/dark cycle) conditions for a 7-day adaptation period. AOM (10 mg/kg) diluted in normal saline was administered to the mice by intraperitoneal injection once per week for 2 consecutive weeks.

Experimental diets and general observation. Seven days after the first AOM injection, the model mice were placed in cages with 12 animals each and divided into the following five groups: Low-dose group (LG), middle-dose group (MG), high-dose group (HG), model control (MC) and normal control (NC). The NC did not receive any treatment and other groups were treated with AOM. After the modeling and immediately after grouping, the mice in the LG, MG and HG groups were administered 2, 4 and 8 g/kg DMT IR-RS aqueous solutions, respectively, once daily for 6 weeks by gavage. The mice in the MC and NC groups were given the same dose of distilled water by gavage. Each group had free access to food and water. The water bottles were filled twice daily. The drinking, diets and activities of the mice were observed daily, and the mice were weighed once per week.

Gastrointestinal emptying of mice. Gastrointestinal emptying was measured as previously described, with slight modifications (25,26). Normal mice (n=5) were fasted for >12 h and had free access to water. The fasted mice were gavaged with different amounts of DMT IR-RS (2, 4 and 8 g/kg) or distilled water (0.4 ml). Two hours later, each mouse was gavaged with 0.4 ml blue ink. The mice were then sacrificed by cervical dislocation. The gut of each mouse was immediately exposed by laparotomy. The guts were expanded and a standard measuring tape was used to measure the distance that the blue ink had advanced in the gut (from the start of the gastric pylorus to the point at which the blue ink ended). Gastrointestinal emptying (%) was calculated according to the following formula: Gastrointestinal emptying (%) = distance advanced/total length of the gut x 100.

Observation of aberrant crypt foci (ACFs). After 8 weeks of gavage, all mice were sacrificed by cervical dislocation, and the colons were removed immediately, flushed with ice-cold normal saline to remove the intestinal contents, cut with scissors longitudinally, placed mucosal side up on strips of filter paper, and another filter paper was used to cover the mucosal surface. Finally, the colons were fixed in 10% buffered formalin for 24 h. Each colon was then cut into 2-3 cm long strips. All segments were put into Petri dishes that contained 0.5% methylene blue solution for 2.5 min and then placed in another Petri dish with a buffer to wash away the excess dye. ACFs were immediately observed under a Gel Imaging System (BIS910; Beijing Dongsheng Innovative Biotechnology Co., Ltd., Beijing, China).

Reverse transcription-polymerase chain reaction (RT-PCR) for the detection of APC, Bax, Bcl-2 and cytochrome c mRNA. The primer sequences for APC, Bax, Bcl-2, cytochrome *c* and GADPH were designed using Primer Premier 5 (Premier Biosoft International, Palo Alto, CA, USA) and synthesized by Shanghai Sangon Biological Engineering Technology & Service Co. Ltd (Shanghai, China); the primer sequences are listed in Table I.

The total RNA of each mouse was extracted from the colon according to the method developed by Yuan *et al* (27) with slight modifications. The RNA extracts were placed in ultra-low temperature freezers (-80˚C) until use. cDNA was synthesized from total RNA with the Revert Aid First Strand cDNA Synthesis kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol, and amplified by PCR. PCR was conducted for 30 cycles. Each amplification cycle consisted of 30 sec at 93˚C for denaturation, 30 sec of cytochrome *c* primer exposure at 59˚C, Bax

Table I. Primer design for RT-PCR.

Gene	Primer sequence (5'-3')	Product size (bp)
Cytochrome *c*	Forward: ACCAAATCTCCACGGTCTGTTC Reverse: GTCTGCCCTTTCTCCCTTCTTC	192
Bax	Forward: ACAGATCATGAAGACAGGGG Reverse: AAAGTAGAAGAGGGCAACCA	298
Bcl-2	Forward: GTCACAGAGGGGCTACGAGT Reverse: GGGTCAGATGGACCACAGG	212
APC	Forward: GGAAGATTGGTTGTAAGTGAAAGGA Reverse: CAAAAAGCAGAGTTAGAACAGGAGG	525
GAPDH	Forward: CCCTTCATTGACCTCAACTAC Reverse: CCACGACTCATACAGCACC	244

APC, adenomatous polyposis coli; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; RT-PCR, reverse transcription-polymerase chain reaction.

Table II. Body weight of each mouse.

	Body weights (g)				
Group	Week 1	Week 2	Week 4	Week 6	Week 8
NC	25.79±0.45	31.56±0.15	36.41±0.07	38.98±0.35	41.34±0.65
MC	25.88±0.15	27.91±0.28[a]	35.61±0.20	39.37±0.27	40.76±0.51
LG	25.14±0.33	27.24±0.45[a]	30.47±0.32[a]	33.65±0.45	38.36±0.08
MG	25.52±0.22	27.53±0.32[a]	26.60±0.26[b]	28.46±0.13[a]	32.25±0.11
HG	25.94±0.25	26.95±0.35[a]	26.75±0.06[b]	27.52±0.09[a]	28.22±0.65[a]

Each result represents the mean value ± standard deviation (n=10). [a]P<0.05 and [b]P<0.01 vs. the NC group. NC, normal control; MC, model control; LG, low-dose group; MG, middle-dose group; HG, high-dose group.

primer exposure at 55˚C, Bcl-2 primer exposure at 55˚C, APC primer exposure at 62˚C or GADPH primer exposure at 55˚C for primer annealing, and 1 min at 72˚C for extension. All PCR products were mixed with 2 μl goldview loading dye (Invitrogen; Thermo Fisher Scientific, Inc.) and subjected to electrophoresis using 1.5% agarose gels containing 0.5 μg/ml ethidium bromide (Invitrogen; Thermo Fisher Scientific, Inc.).

Statistical analysis. All experiments were repeated ≥10 times. All experimental data are expressed as the mean ± standard deviation. Differences between test subjects and model controls were evaluated using Student's t-tests. P<0.05 was considered to indicate a statistically significant difference.

Results and Discussion

Observations of the daily activities and body weights of the mice. During the experiment, each group of mice exhibited normal eating, drinking and activity. The changes in body weight in each group of mice during the experiment were analyzed, and the results are summarized in Table II. The results revealed no significant differences (P>0.05) in initial body weight (25.65±0.33 g) prior to the AOM injections between the five experimental groups. After the AOM injections (week 2), the body weights of the mice in the NC group increased by 22.4%, while the other four groups of mice gained less weight than did the normal group, and there were no significant difference between the four experimental groups. After 6 weeks of feeding with RS solution (LG, 2 g/kg; MG, 4 g/kg; HG, 8 g/kg), the gains in body weight of the mice in the LG, MG and HG groups were lower than those of the NC group, and the weight gain of the HG group was significantly different from that of the NC group (P<0.05). The body weights of the MC, LG and MG groups increased by 57.5, 52.7 and 26.4%, respectively, and there were no significant differences relative to the NC group (P>0.05).

The results revealed that the body weights of the MC group increased rapidly and were not significantly different (P>0.05) from those of the NC group at 8 weeks. This finding indicates that the inchoate colon cancer induced by AOM had no clear effect on the body weights of the mice. However, the body weight gains in the RS-treated groups exhibited a dose-dependent reduction, which indicates that RS effectively controlled the increases in the body weights of the mice. In addition, some differences were observed in the food intake of the mice in different treatment groups. The amount of

Table III. Influence of RS on colonic ACFs in mice.

Group	ACF incidence	Number of ACFs	Number of aberrant crypts	Mean number of aberrant crypts
NC	0/16	0	0	0
MC	16/16	11±2	32±5	3.0±0.13
LG	16/16	9±1	25±5	2.9±0.45
MG	16/16	8±1[a]	26±4	3.2±0.25
HG	16/16	7±1[b]	20±3[b]	3.0±0.16

Values are presented as the mean value ± the standard deviation (n=10). [a]P<0.05 and [b]P<0.01 vs. the MC group. RS, resistant starch; ACF, aberrant crypt foci; NC, normal control; MC, model control; LG, low-dose group; MG, middle-dose group; HG, high-dose group.

food intake decreased in mice after feeding with the RS solution, and the NC and MC groups exhibited normal eating. RS does not readily degrade in the small intestine; thus, RS can reduce the postprandial glycemic response and food intake and increase satiety (28). RS can also be fermented to short-chain fatty acids by microorganisms in the colon (29). Following the rapid absorption of short-chain fatty acids by colorectal tissue, energy is stored and the osmotic pressure is reduced; short-chain fatty acids are important in maintaining the morphology and function of the normal colon and colonic epithelial cell function (30). Therefore, RS can reduce the intestinal pH and the quantity of carcinogens, and promote the absorption of trace elements.

Effects of RS on gastrointestinal emptying. Gastrointestinal emptying plays different and important roles in accommodating gastrointestinal function (31). As shown in Fig. 1, the gastrointestinal emptying rate of the normal mice increased following DMT IR-RS gavage. The gastrointestinal emptying rate of the high-dose group (38.5±0.9%) was significantly different from that of the mice treated with distilled water only (P<0.01). Treatment with 2 or 4 g/kg IR-RS did not markedly affect gastrointestinal emptying rates, which indicates that a certain amount of RS helps to improve gastrointestinal emptying and promote gastrointestinal motility. These results should aid the analysis of the role of RS in gastrointestinal motility and may provide a new strategy for future studies of colon cancer.

Effects of RS on ACFs in the colons of mice. Following the intraperitoneal injection of AOM, the incidence of ACFs in the mice was 100%. The ACF configurations are presented in Fig. 2A. The ACF incidence in the NC group was 0%, and the configuration is shown in Fig. 2B. The ACF incidence revealed that the colon cancer model was successful. In comparison with the NC group, the ACF characteristics of the other groups included comparatively large crypt foci, deep staining, oval cavity recesses and additional layers of epithelial cells.

Table III summarizes the effect of RS on the ACFs of the mice. The numbers of ACFs in the RS-treated groups exhibited a dose-dependent reduction compared with those in the MC group, while the MG and HG groups had significantly different numbers of ACFs from the MC group (P<0.05). In the RS-treated groups, the numbers of aberrant crypts in the LG group did not differ from those of the MG group (P>0.05),

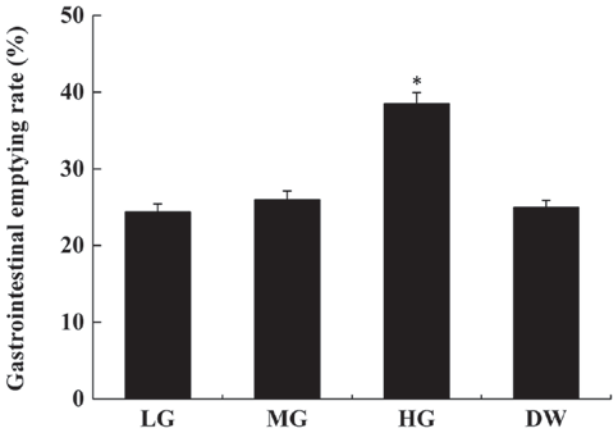

Figure 1. Influence of RS on gastrointestinal emptying rate. Results are expressed as the mean ± standard deviation. *P<0.01 vs. the DW group. LG, low-dose group; MG, middle-dose group; HG, high-dose group; DW, distilled water group.

and they were also not significantly different from those in the MC group. The number of aberrant crypts in the HG group was significantly different from that of the MC group (P<0.01). The average numbers of aberrant crypts in each ACF of the RS-treated groups were not dose-dependent, and the differences were not statistically significant. These results indicate that a specific diet of RS can effectively reduce the numbers of ACFs and aberrant crypts.

ACFs were first proposed by Bird *et al* (32) in 1987 and can be observed in CRC when viewing early colorectal mucosal lesions under an optical microscope. ACFs typically consist of one, several or even hundreds of abnormal aberrant crypts (33). The mechanism of the effect of RS on ACFs is mediated by RS inducing increases in diet and intestinal emptying rate, reducing fecal pH, and increasing the levels of intestinal short chain fatty acids, which dilutes carcinogens, accelerates their excretion, and reduces their likelihood of coming into contact with the intestinal epithelium, and thereby suppresses the occurrence of ACFs (34).

Effect of RS on the expression of APC, Bax, cytochrome c and Bcl-2 genes in the colon. RT-PCR assays were used to detect the specific mRNA expression levels of APC, Bax, cytochrome *c* and Bcl-2, and the results are shown in Fig. 3. GAPDH served as an internal reference for the calculation of the relative expression levels of the mRNAs. At 6 weeks of RS

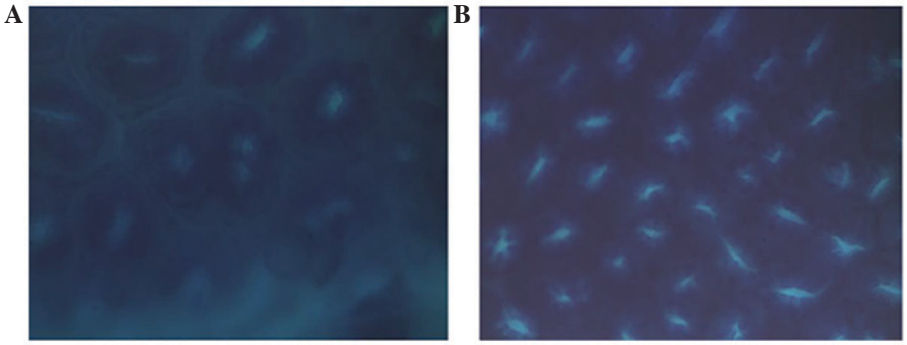

Figure 2. Shapes of the ACFs in the mice examined using light microscopy. (A) ACF configurations induced by the injection of azoxymethane, and (B) the NC group (methylene blue staining). The ACF incidence in the NC group was 0%. Magnification, x100. ACF, aberrant crypt foci; NC, normal control.

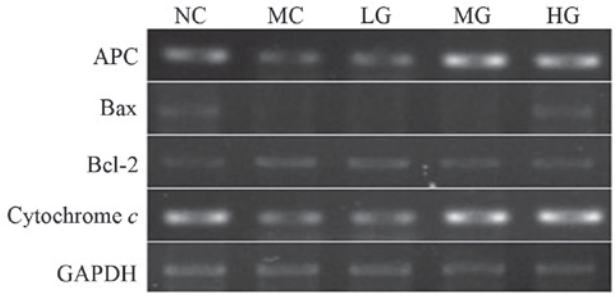

Figure 3. Expression of APC, Bax, cytochrome *c* and Bcl-2 in the colon. Reverse transcription-polymerase chain reaction assays were used to detect the specific expression levels of APC, Bax, cytochrome *c* and Bcl-2 genes. APC, adenomatous polyposis coli; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; NC, normal control; MC, model control; LG, low-dose group; MG, middle-dose group; HG, high-dose group.

gavage, the expression levels of the amplification products of APC, Bax and cytochrome *c* in the NC group were higher than those of the MC group. It was also observed that RS treatment promoted the expression of APC, Bax and cytochrome *c* and that these increases exhibited a trend toward being dose-dependent. By contrast, the expression of the anti-apoptotic gene Bcl-2 in the NC group was lower than that of the MC group. The results showed that RS treatment inhibited the expression of Bcl-2, and these reductions exhibited a trend towards being dose-dependent.

The occurrence of CRC followed the 'adenoma to adenocarcinoma' law of development that was first proposed by Muto *et al* (35) in 1975. Fearon and Vogelstein (36) subsequently noted that the progression of 'adenoma to adenocarcinoma' involves changes in suppressor genes and oncogenes. Additionally, the mechanism of CRC also involves changes in signaling pathways. The occurrence of CRC is caused by changes in these genes and their long-term accumulation, and its mechanism is essentially a polygenic multi-step process. The APC gene is an important tumor suppressor gene in colon cancer and is located on human chromosome 5q21 (9). Deletions or mutations of the APC gene are closely associated with the occurrence of early CRC; 85% of colon cancers are accompanied by deletions and inactivation of the APC gene. The protein product of the APC gene typically combines with β-connexin protein (β-catenin) to control excessive cell proliferation. When APC is mutated, its ability to regulate cell proliferation is reduced. Therefore, the APC-β-catenin pathway is considered to be an important therapeutic target for early colon cancer (9,37). In the present study, it was found that the expression of APC in the MC group was lower than that in the NC group; however, after 6 weeks of gavage treatment, APC expression was higher than that of the MC group and increased in a dose-dependent manner. These results may be due to the ability of dietary RS to reduce the methylation of the APC gene, enabling APC and β-catenin to bind and thereby inhibit excessive cell proliferation.

The Bcl-2 family, which includes Bcl-2 and Bax genes, plays an important role in the regulation of apoptosis (38). In normal colonic mucosa, the Bcl-2 gene was found to be expressed in the mucosal crypts, and no cell apoptosis occurred, whereas the Bax gene was expressed in epithelial cells and promoted cell apoptosis. These findings indicate that these genes coordinate in normal mucosa to control cell proliferation and apoptosis so that the epithelial cells, which are produced by the mucosal crypts can migrate upward until death due to cellular aging (39). It has been found that Bcl-2 can antagonize the pro-apoptotic effect of Bax on cells and that the Bcl-2/Bax ratio is closely associated with apoptosis; indeed, this ratio is considered to be a molecular switch for the initiation of apoptosis (13). The present study found that the expression of the Bax gene in the MC group was weaker than that in the NC group, and that the expression of the Bcl-2 gene in the MC group was stronger than that in the NC group, in which the Bcl-2/Bax ratio was relatively high. However, after 6 weeks of gavage treatment, Bax gene expression increased, and Bcl-2 gene expression decreased to produce a relatively low Bcl-2/Bax ratio. These results demonstrate that RS improved the abnormal expression of the Bcl-2 family of pro- and anti-apoptotic genes in the colon mucosa to bring the Bcl-2/Bax ratio close to that of the MC group and induce apoptosis.

Cytochrome *c* is a water-soluble protein that regulates the energy metabolism of the mitochondria and also regulates apoptosis (15). Cytochrome *c* plays a negative regulatory role and inhibits the growth of tumor cells in the process of tumor development. The mechanism of action of cytochrome *c* is mediated by apoptotic signal conduction and amplification that results in the regulation of apoptosis (12). Cytochrome *c* is closely associated with the Bcl-2 family. The Bcl-2 family can participate in the process of cytochrome *c* release and the

formation of an integral membrane protein. Bcl-2 stabilizes the mitochondrial membrane and suppresses the release and activation of cytochrome *c* and caspase, which changes the oxidation-reduction reaction within the nucleus and ultimately inhibits apoptosis. It has been reported that the content of cytochrome *c* in colorectal tissues adjacent to cancerous tissue is significantly higher than that in the cancerous tissues, which suggests that tumors may be associated with inhibition of the release of cytochrome *c*. In the present study, it was found that the expression of cytochrome *c* mRNA in the MC group was weaker than that in the NC group. However, after 6 weeks of gavage treatment, cytochrome *c* mRNA expression increased, and this result demonstrates that RS effectively increased the release of cytochrome *c* from the colon mucosa cells. The results for APC, Bax, cytochrome *c* and Bcl-2 expression levels in the colon indicate that DMT IR-RS promoted the expression of APC, Bax and cytochrome *c* and inhibited the expression of Bcl-2. These findings indicate that DMT IR-RS may induce colonic epithelial cell apoptosis and decrease the effects of colon cancer in mice.

In conclusion, the present study demonstrated that the DMT IR-RS product can induce apoptosis and has beneficial health effects in mice with AOM-induced early colon cancer. The body weight gains, gastrointestinal emptying rates and numbers of ACFs in the RS-treated groups decreased dose-dependently. In addition, DMT IR-RS diets may induce the occurrence of apoptosis and reduce the effects of colon cancer in mice. RS is difficult to digest in the small intestine and is fermented by microorganisms to increase the amounts of short-chain fatty acids, particularly butyric acid. Butyric acid can reduce carcinogen levels in the gut and inhibit tumor cells (40). In our future studies, we will examine how RS changes the intestinal microbial system to affect the generation and discharge of intestinal toxins and how toxins affect glucose metabolism in mice.

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