

RESEARCH ARTICLE

Suppression of β -catenin and Cyclooxygenase-2 Expression and Cell Proliferation in Azoxymethane-Induced Colonic Cancer in Rats by Rice Bran Phytic Acid (PA)

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Abstract

Background: Phytic acid (PA) is a polyphosphorylated carbohydrate that can be found in high amounts in most cereals, legumes, nut oil, seeds and soy beans. It has been suggested to play a significant role in inhibition of colorectal cancer. This study was conducted to investigate expression changes of β -catenin and cyclooxygenase-2 (COX-2) and cell proliferation in the adenoma-carcinoma sequence after treatment with rice bran PA by immunocytochemistry. **Materials and Methods:** Seventy-two male Sprague-Dawley rats were divided into 6 equal groups with 12 rats in each group. For cancer induction two intraperitoneal injections of azoxymethane (AOM) were given at 15 mg/kg bodyweight over a 2-weeks period. During the post initiation phase, two different concentrations of PA, 0.2% (w/v) and 0.5% (w/v) were administered in the diet. **Results:** Results of β -catenin, COX-2 expressions and cell proliferation of Ki-67 showed a significant contribution in colonic cancer progression. For β -catenin and COX-2 expression, there was a significant difference between groups at $p < 0.05$. With Ki-67, there was a statistically significant lowering the proliferating index as compared to AOM alone ($p < 0.05$). A significant positive correlation ($p = 0.01$) was noted between COX-2 expression and proliferation. Total β -catenin also demonstrated a significant positive linear relationship with total COX-2 ($p = 0.044$). **Conclusions:** This study indicated potential value of PA extracted from rice bran in reducing colonic cancer risk in rats.

Keywords: Phytic acid - azoxymethane - colon cancer - β -catenin - COX-2 - Ki-67 - rat model

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Introduction

Colorectal cancer as one of the types of cancer is ranked as the second most common cancer among men and third most common cancer among women (American Cancer Society, 2008). According to Ministry of Health Malaysia (2007), colorectal cancer is also the second most common cancer in Malaysia with the percentage of 18.1% after breast. It is ranked as the first most common among male (14.6%) and the second among female (10%) (MOH, 2007). Colorectal carcinogenesis is caused by a defined set of molecular events (Tejpar and Cutsem, 2002). Thus, several lines of evidence indicate that the molecular events contributing to the progression of colon cancer begins by initiation and probably obligatory activation of β -catenin oncogene (Polakis et al., 1999). This can occur by the loss of its negative regulator of the adenomatous polyposis coli (APC) protein, or by mutation in the β -catenin gene that result in a more stable protein product (Clevers, 2006). β -catenin acts as an intracellular signal transducer in the Wnt signaling pathway. Wnt signaling pathway also known as APC/ β -catenin signaling pathway is directly related to colorectal carcinogenesis (Chung,

2000) thus plays important roles in development, cellular proliferation and differentiation (Ebert et al., 2002).

Cyclooxygenase- 2 (COX-2), an inducible prostaglandin G/H synthase is not detectable in most healthy tissue but is produce in response to various stimuli and is involved in the production of prostaglandins (PGs) from free arachidonic acid (Zerkowski et al., 2007). COX-2 protein was shown over expressed in colorectal cancer tissue and suggested to play an important role in the early phase of tumor progression (Lim et al., 2007). It is also believed to enhance tumor promotion by promoting cell proliferation, angiogenesis and apoptotic evasion, stimulating tumor metastasis and decreasing immune surveillance (Eisinger et al., 2007) as well as induce inflammation (Crofford et al., 1994). Thus, COX-2 is an attractive therapeutic target of colorectal cancer (Arber 2008). The Ki-67 is a nuclear and nucleolar protein, which is tightly associated with somatic cell proliferation (Mobius et al., 2005). Ki-67 immunoreactivity in cancers attracts attention, as Ki-67 reflects cell proliferation.

Treatment being used for cancer such as chemotherapeutic agents tends to increase the possibilities of getting unwanted side effects such as nausea and

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vomiting, fatigue, diarrhea, constipation, hair loss and fever (American Cancer Society, 2009). Due to the noticeable lack of side effects, natural products have received great attention in cancer prevention and treatment. In the present study, the effect of phytic acid (PA) extracted from rice bran (EPA) on the expression of β -catenin, COX-2 and cell proliferation of Ki-67 was examined by using adenoma and adenocarcinoma samples. The association between β -catenin, COX-2 and Ki-67 was also investigated. By observing this therefore, it may be postulated as a key target for prevention of colon cancer, hence natural chemoprevention agent from Malaysian sources can be developed. Apart from its medicinal values, it also can improve the economic value of rice bran, which is normally discarded as by-product of rice production.

Materials and Methods

Animals and diets

Seventy two male Sprague Dawley rats (*Rattus Norvegicus*), weighing about 95 g, purchased from the Animal Colony Unit, Faculty of Veterinary Medicines, UPM, Serdang, Selangor were used as experimental colon cancer model. They were housed individually in a fully ventilated room under 12 hours light-dark cycles in a temperature-controlled room (25-27 °C). This study was carried out according to the guidelines approved by the Animal Care and Use Committee of the Faculty of Medicine and Health Sciences, Universiti Putra Malaysia (UPM) (Reference no: UPM/FPSK/PADS/BR-UUH/00289). A colonic carcinogen, azoxymethane (AOM) was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Preparation of rice bran PA has been described in previous study (Norazalina et al., 2010).

Induction of colon carcinogenesis

Azoxymethane (AOM), a specific carcinogen was diluted in 0.9% (v/v) saline. For two weeks, AOM (15 mg/kg body weight) was given intraperitoneally (i.p.) to animals once a week to induce colonic tumors (Bird, 1998).

Experimental design

The experimental design is summarized in Figure 1. A total of 72 rats were acclimatized for 1 week on receiving a standard commercial feed (Gold Coin Rat Chow, Australia) and ad libitum. They were divided randomly into six groups. Each group comprises of 12 animals and were treated as follows: Group 1: Normal (NC). Group 2: AOM alone (PC). Group 3: AOM+0.2% (w/v) commercial phytic acid (CPA). Group 4: AOM+0.5% (w/v) commercial phytic acid (CPA). Group 5: AOM+0.2% (w/v) extracted phytic acid (EPA). Group 6: AOM+0.5% (w/v) extracted phytic acid (EPA)

After acclimatization, the rats in groups 2-6 were intended for carcinogen treatment whereas the rats' in-group 1 received an equal volume of normal saline and served as vehicle control. Each rat in groups 3-6 were given two different concentration of PA (0.2% and 0.5% w/v) in drinking water during post-initiation

phase (beginning 1 week after carcinogen treatment) of carcinogenesis. These levels were selected based on published research by Ullah and Shamsuddin (1990). The rat in group 1 had free access to water and the amounts of water consumed were also measured daily. All rats were carefully observed daily and their weights were recorded once a week using electronic balance.

Termination of experiment

After 20 weeks of the respective treatment, all the rats were sacrificed under ether anesthesia. Colon tissue were collected, flushed with saline and slit opened longitudinally from cecum to anal. The number, size and infiltrate tumors were observed and the grades of the tumors were classified into adenoma and adenocarcinoma. The finding on the tumor incidence and tumor multiplicity has been published earlier (Norazalina et al., 2010). All tissues samples were submerged overnight in 10% (v/v) buffered formalin, embedded in paraffin wax, and then sliced for 3 μ m in thickness in an automatic tissue processor machine for immunohistochemical staining. The expression of immunohistochemical staining of β -catenin, COX-2 and Ki-67 antigen were compared between different grades of tumors in different groups of treatment.

Immunohistochemical staining of β -catenin, COX-2 and Ki-67 antigen

The paraffin-embedded sections were heated in a hot air oven at 60 °C for an hour. Then, the sections were deparaffinized and dehydrated by sequential immersion in two times of xylene, graded alcohol (100%, 95%, 80%, and 70%) for 3 minutes each, followed by a rinse with distilled water. Dewaxed sections then were immersed in boiling 10 mM Tris buffer, 1 mM EDTA (pH 9.0) for 20 minutes to expose the antigenic sites that might be hindered during the embedding process and to allow access of antibodies to the cell content. The sections were allowed to cool at room temperature before incubated with 3% (v/v) hydrogen peroxide (10 minutes) to quench endogenous peroxide. After washing with Tris-buffered saline in 0.1% Tween-20 (TBST), pH 7.6 the sections were further blocked using 0.01% d-biotin followed by 3% bovine serum albumin (BSA) for an hour to reduce the non-specific antibody binding. The sections then were incubated with primary antibodies for an hour at room temperature or overnight (~18 hours) at 4 °C. Primary antibodies used for β -catenin, COX-2 and Ki-67 were monoclonal mouse anti-rat β -catenin antibody (catalogue number 610153, clone 14, Transduction Laboratories, Lexington, KY), monoclonal mouse anti-rat COX-2 antibody (catalogue number RB-9072, clone SP21, Labvision Corporation., USA) and monoclonal mouse anti-rat Ki-67 antibody (catalogue number RM-9106, clone SP6, Labvision Corporation, USA), respectively. All primary antibodies were diluted to 1: 100 before used. For negative control purposes, primary antibodies were omitted. The colorectal carcinoma tissue and HeLa cell (cervical cancer cell line) were used as internal positive control for β -catenin, colorectal carcinoma tissue for COX-2 and lymphoid tissue of the colon for Ki-67.

Immunostaining was performed using LSAB[®]2 System-HRP kit (DAKO, Carpinteria, CA, USA) in room temperature according to the manufacturers instruction. After rinsing with Tris-buffered saline (TBS), biotin-conjugated secondary antibody was applied for at least 30 minutes followed by incubation with peroxides-conjugated streptavidin for a another 30 minutes at room temperature. All steps were followed by washing in TBS. Peroxidase activities were detected by incubating the samples with 3, 3'-Diaminobenzidine (DAB) chromogen substrate which resulted in the presence of a brown precipitate. Finally, the sections were counterstained with hematoxylin and mounted.

Evaluation of immunohistochemical staining

For the scoring system, seven areas in the sections stained with respective antibody were chosen randomly by using a 4x objective magnification. A modified semi-quantitative scoring system used to evaluate the staining by all the antibodies was followed as previous study (Kohno et al., 2005). The percentage of positive cells was evaluated using the following scale: 0, no staining of cells in any field; 1=positive staining in 1-25%; 2=positive staining in 26-50%; 3=positive staining in 51-75%; and 4=positive staining in 76-100%. While the strength of staining intensity was evaluated using the following range: 0, no staining of cells; 1+, mild staining; 2+, moderate staining and 3+, strong staining. The total score was generated by adding the score for percentage of positive cells and the strength of staining intensity. Consequently, the minimum and maximum score for areas were 0 and 7 respectively.

Statistical analysis

The data are presented as means±standard deviation (SD). In immunohistochemical analysis, since the total score was not distributed, the analysis between antigens was analyzed using Kruskal-Wallis test. For the comparison between groups of treatment, Mann-Whitney U-test was applied. Spearman rank correlation test was used to analyze the correlation among the expression of biomolecules. A mean difference is considered significant when $p < 0.05$. Statistical analysis was performed using Statistical Package of Social Science (SPSS) for Window version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Table 1 summarized the immunohistochemical staining for β -catenin, COX-2 and Ki-67. β -catenin was mainly localized at the membranes of the cell borders in normal epithelial cells. Negative control sections showed no positive staining when the primary antibody was omitted (result not shown). Strong β -catenin expression was seen in the cell membrane and cytoplasm of colon mucosa carcinoma cells in Group 2: AOM alone (untreated) (Figure 2: 1A). The intensity was relatively stronger than Group AOM+CPA and Group AOM+EPA (Figure 2; 1B, 1C). Table 2 presented a comparison of immunohistochemical staining for the expression of β -catenin compared between groups. There was a significant difference between groups

of 0.2% EPA (w/v) and 0.5% CPA (w/v) as compared to AOM alone. Though, there was no significance effect between 0.5% EPA (w/v) and 0.2% EPA (w/v) compared to AOM alone. However, there was a significant difference ($p < 0.05$) between groups of treatment 0.2% EPA (w/v) and 0.2% CPA (w/v), 0.5% EPA (w/v) and 0.5% CPA (w/v), 0.5% EPA (w/v) and 0.2% CPA (w/v), 0.2% EPA (w/v) and 0.5% CPA (w/v).

COX-2 expression was observed in the tumor colonic tissues, where it was located within the cytoplasm. The weak COX-2 immunoreactivity was found in the cytoplasm of normal colonic mucosa tissues (result not shown). Strong COX-2 immunoreactivity was found in the adenocarcinoma cytoplasm (Fig 2: 2A) whereas the immunoreactivity was weaker in the cytoplasm of normal colonic mucosa tissues and in treatment groups of PA (Figure 2: 2B, 2C). There was a statistically significant difference between groups in COX-2 expression ($p = 0.000$) (Table 3). As presented in Table 3, 0.2% (w/v) CPA, 0.5% (w/v) CPA, 0.2% EPA (w/v) and 0.5% EPA (w/v) show a significant difference when compared to AOM alone. However, there was no significant effect between different percentages of PA given.

The immunoreactivity of proliferative activity of colon cancer is defined by Ki-67. The staining produced diffuse, strong nuclear stain, which was easily identified (Figure 2: 3A-3C), whereas the cytoplasm and the cell membrane remained unstained by Ki-67. Negative control sections showed no positive staining when the primary antibody step was omitted (result not shown). The cell was

Table 1. Mean Score for the Expression of β -catenin, COX-2 and Ki-67 in Colonic Tissue

| | Groups | | | | | p |
|------------------|-----------|----------------|----------------|----------------|----------------|-------|
| | AOM alone | AOM+ | | AOM+ | | |
| | | 0.2% CPA (w/v) | 0.5% CPA (w/v) | 0.2% EPA (w/v) | 0.5% EPA (w/v) | |
| Ki-67 | 100.2 | 74.8 | 65.2 | 72.6 | 64.3 | 0.001 |
| β -catenin | 94.8 | 48.8 | 59.1 | 82.1 | 92.6 | 0 |
| COX-2 | 101.9 | 72.1 | 69 | 60.5 | 74.7 | 0 |

*CPA=Commercial Phytic Acid; EPA=Extract Phytic acid; AOM=Azoxymethane. Each value expressed as mean±SD (n=6). Groups: AOM alone=Azoxymethane alone, AOM+0.2% (w/v) CPA=Azoxymethane+0.2% (w/v) Commercial Phytic Acid, AOM+0.5% (w/v) CPA=Azoxymethane+0.5% (w/v) Commercial Phytic Acid, AOM+0.2% (w/v) EPA=Azoxymethane+0.2% (w/v) Extract Phytic Acid, AOM+0.5% (w/v) EPA=Azoxymethane+0.5% (w/v) Extract Phytic Acid, Normal

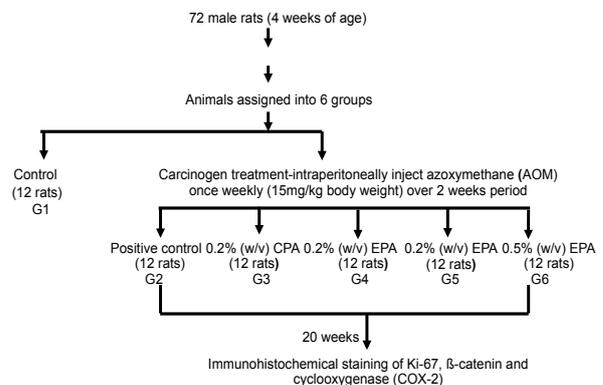


Figure 1. Experimental Protocol (CPA, commercial phytic acid; EPA, extract phytic acid)

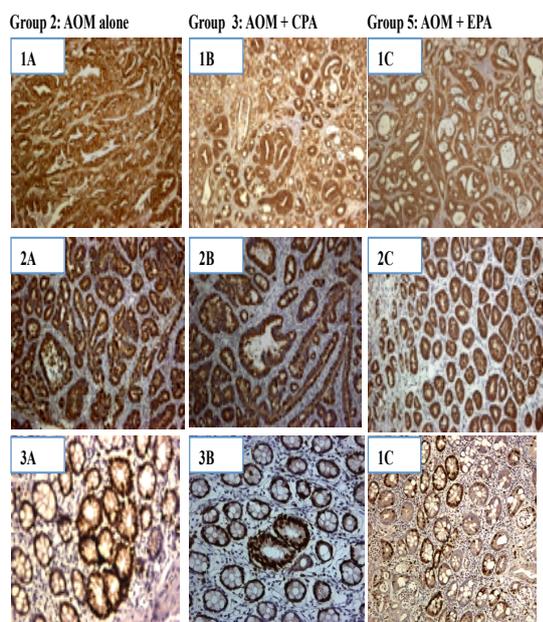


Figure 2. Immunohistochemical staining of β -catenin and COX-2 and Ki-67 in AOM Alone (untreated) (Group 2) Compared to Treatment Groups of Rats with Commercial Phytic Acid (CPA) (Group 3) and Extracted Phytic Acid (EPA) (Group 5). β -catenin immunoreactivity showed membrane and cytoplasmic staining in representative Group 2, Group 3 and Group 5 (1A, 1B, 1C). Expression of COX-2 in cytoplasm of colonic cells: strong COX-2 in Group 2 (2A), weaker staining of COX-2 in Group 3 (2B), and weakest staining of COX-2 in Group 5 (2C) and immunoreactivity of Ki-67 in colonic sections showed a sequence of reduction in proliferative activity of the nuclei (3A, 3B and 3C)

Table 2. Comparison Between Groups of Treatment in β -catenin Scores

| Group | Mean Rank | Sum of rank | p |
|--------------------|-----------|-------------|-------|
| AOM alone | 41.6 | 1706 | 0.00* |
| AOM+0.2% (w/v) CPA | 22 | 572 | |
| AOM alone | 41.8 | 1712 | 0.00* |
| AOM+0.5% (w/v) CPA | 25.1 | 703 | |
| AOM alone | 39.7 | 1628.5 | 0.15 |
| AOM+0.2% (w/v) EPA | 33.5 | 1072.5 | |
| AOM alone | 34.7 | 1424 | 0.89 |
| AOM+0.5% (w/v) EPA | 34.2 | 922 | |
| AOM+0.2% (w/v) CPA | 25.1 | 651.5 | 0.25 |
| AOM+0.5% (w/v) CPA | 29.8 | 833.5 | |
| AOM+0.2% (w/v) EPA | 28.1 | 898.5 | 0.28 |
| AOM+0.5% (w/v) EPA | 32.3 | 871.5 | |
| AOM+0.2% (w/v) CPA | 22.5 | 586 | 0.00* |
| AOM+0.2% (w/v) EPA | 35.2 | 1125 | |
| AOM+0.5% (w/v) CPA | 22.1 | 619.5 | 0.00* |
| AOM+0.5% (w/v) EPA | 34.1 | 920.5 | |
| AOM+0.2% (w/v) CPA | 19.7 | 512 | 0.00* |
| AOM+0.5% (w/v) EPA | 34 | 919 | |
| AOM+0.2% (w/v) EPA | 34.8 | 1113.5 | 0.00* |
| AOM+0.5% (w/v) CPA | 25.6 | 716.5 | |

*CPA=Commercial Phytic Acid; EPA=Extract Phytic acid; AOM= Azoxymethane. The statistical significance of the differences was analyzed using Mann-Whitney's U-test. p<0.05 indicates statistical significance. Groups: AOM alone=Azoxymethane alone, AOM+0.2% (w/v) CPA=Azoxymethane+0.2% (w/v) Commercial Phytic Acid, AOM+0.5% (w/v) CPA=Azoxymethane+0.5% (w/v) Commercial Phytic Acid, AOM+0.2% (w/v) EPA=Azoxymethane+0.2% (w/v) Extract Phytic Acid, AOM+0.5% (w/v) EPA= Azoxymethane+0.5% (w/v) Extract Phytic Acid

considered positive if there was a clearly detectable brown color in the nucleus. Cytoplasmic staining was considered as non-specific and was not taken into consideration. As shown in Table 4, there was a significant difference

Table 3. Comparison Between Groups of Treatment in COX-2 Scores

| Group | Mean Rank | Sum of rank | p |
|--------------------|-----------|-------------|-------|
| AOM alone | 39.7 | 652.5 | 0.00* |
| AOM+0.2% (w/v) CPA | 25.1 | 625.5 | |
| AOM alone | 41.7 | 707.5 | 0.00* |
| AOM+0.5% (w/v) CPA | 25.3 | 1707.5 | |
| AOM alone | 44.9 | 1841.5 | 0.00* |
| AOM+0.2% (w/v) EPA | 26.9 | 859.5 | |
| AOM alone | 38.7 | 1585 | 0.02* |
| AOM+0.5% (w/v) EPA | 28.2 | 761 | |
| AOM+0.2% (w/v) CPA | 28.2 | 732 | 0.75 |
| AOM+0.5% (w/v) CPA | 26.9 | 753 | |
| AOM+0.2% (w/v) EPA | 27.7 | 887.5 | 0.24 |
| AOM+0.5% (w/v) EPA | 32.7 | 882.5 | |
| AOM+0.2% (w/v) CPA | 32.5 | 845 | 0.2 |
| AOM+0.2% (w/v) EPA | 27.1 | 866 | |
| AOM+0.5% (w/v) CPA | 27.4 | 786 | 0.75 |
| AOM+0.5% (w/v) EPA | 28.7 | 774 | |
| AOM+0.2% (w/v) CPA | 26.8 | 697 | 0.93 |
| AOM+0.5% (w/v) EPA | 27.2 | 734 | |
| AOM+0.2% (w/v) EPA | 28.3 | 907 | 0.23 |
| AOM+0.5% (w/v) CPA | 33 | 923 | |

*CPA=Commercial Phytic Acid; EPA=Extract Phytic acid; AOM= Azoxymethane. The Statistical significance of the differences was analyzed using Mann-Whitney's U-test. p<0.05 indicates statistical significance. Groups: AOM alone=Azoxymethane alone, AOM+0.2% (w/v) CPA= Azoxymethane+0.2% (w/v) Commercial Phytic Acid, AOM+0.5% (w/v) CPA=Azoxymethane+0.5% (w/v) Commercial Phytic Acid, AOM+0.2% (w/v) EPA= Azoxymethane+0.2% (w/v) Extract Phytic Acid, AOM+0.5% (w/v) EPA= Azoxymethane+0.5% (w/v) Extract Phytic Acid

Table 4. Comparison Between Groups of Treatment in Ki67 Scores

| Group | Mean Rank | Sum of rank | p |
|--------------------|-----------|-------------|-------|
| AAOM alone | 38.6 | 1583.5 | 0.01* |
| AOM+0.2% (w/v) CPA | 26.7 | 694.5 | |
| AOM alone | 41.6 | 1706.5 | 0.00* |
| AOM+0.5% (w/v) CPA | 25.3 | 708.5 | |
| AOM alone | 42.4 | 1737.5 | 0.01* |
| AOM+0.2% (w/v) EPA | 30.1 | 963.5 | |
| AOM alone | 40.6 | 1664.5 | 0.00* |
| AOM+0.5% (w/v) EPA | 25.2 | 681.5 | |
| AOM+0.2% (w/v) CPA | 29.4 | 763 | 0.37 |
| AOM+0.5% (w/v) CPA | 25.8 | 722 | |
| AOM+0.2% (w/v) EPA | 31.3 | 1002.5 | 0.5 |
| AOM+0.5% (w/v) EPA | 28.4 | 767.5 | |
| AOM+0.2% (w/v) CPA | 30.2 | 784 | 0.78 |
| AOM+0.2% (w/v) EPA | 29 | 927 | |
| AOM+0.5% (w/v) CPA | 28.4 | 796 | 0.83 |
| AOM+0.5% (w/v) EPA | 27.6 | 744 | |
| AOM+0.2% (w/v) CPA | 29 | 755 | 0.32 |
| AOM+0.5% (w/v) EPA | 25 | 676 | |
| AOM+0.2% (w/v) EPA | 31.7 | 1013.5 | 0.56 |
| AOM+0.5% (w/v) CPA | 29.2 | 816 | |

*CPA=Commercial Phytic Acid; EPA=Extract Phytic acid; AOM= Azoxymethane. The statistical significance of the differences was analyzed using Mann-Whitney's U-test. p<0.05 indicates statistical significance. Groups: AOM alone=Azoxymethane alone, AOM+0.2% (w/v) CPA= Azoxymethane+0.2% w/v Commercial Phytic Acid, AOM+0.5% (w/v) CPA=Azoxymethane+0.5% (w/v) Commercial Phytic Acid, AOM+0.2% (w/v) EPA= Azoxymethane+0.2% (w/v) Extract Phytic Acid, AOM+0.5% (w/v) EPA= Azoxymethane+0.5% (w/v) Extract Phytic Acid

Table 5. Correlation among the Total Scores of Ki67, β -catenin and COX-2

| | | Ki-67 | β -catenin | COX-2 |
|------------------|------|-------|------------------|--------|
| Ki-67 | C.C. | | 0.03 | 0.21** |
| | p | | 0.76 | 0.01 |
| β -catenin | C.C. | 0.03 | | 0.16* |
| | p | 0.76 | | 0.04 |
| COX-2 | C.C. | 0.21 | 0.16* | |
| | p | 0.01 | 0.04 | |

* $p < 0.001$, and ** $p < 0.05$ The correlation was analyzed by using spearman rank correlation test. C.C.=Correlation Coefficient

in lowering the proliferating index between treatment groups as compared to AOM alone. However, there was no significant difference observed when 0.2% CPA (w/v) compared to 0.5% (w/v) CPA, 0.2% (w/v) EPA compared to 0.5% (w/v) EPA, 0.2% (w/v) CPA compared to 0.2% (w/v) EPA, 0.5% (w/v) CPA compared to EPA w/v, 0.2% (w/v) CPA compared to 0.5% (w/v) EPA and between 0.2% EPA (w/v) compared to 0.5% CPA (w/v).

The correlation between total scores of β -catenin, COX-2 and Ki-67 were presented in Table 5. By using Spearman rank correlation test, the result showed that there was a significant ($p=0.010$) positive correlation between proliferation of Ki-67 and COX-2 expression. A positive linear relationship was found between total Ki67 and β -catenin but these relationships were not statistically significant. Total β -catenin also had a positive linear relationship with total COX-2 ($p=0.44$).

Discussion

In this study, we investigated the changes in the expression of β -catenin, COX-2 and cell proliferation using Ki-67 which contribute in the sequence of colon cancer by immunohistochemical analyses. This technique has several advantages over the other techniques such as simplicity of the methods, maintenance of cellular, tissue architecture and the rapidity of results (Oshima et al., 2005).

β -catenin is a key regulator and an important element of the cadherin-mediated cell-cell adhesion system. The adenomatous polyposis coli (APC) tumor suppression protein, which associates with β -catenin (Rubinfeld et al., 1996) is involved in down-regulation of β -catenin together with serine-threonine glycogen synthase kinase (GSK)-3 β (Munemetsu et al., 1995, Yost et al., 1996). Mutations in the GSK-3 β as APC mutations, caused stabilization of β -catenin in the cytoplasm and induced constitutive transcriptional activation with Tcf-4, a member of the Tcf family of DNA-binding protein. Therefore, activation of the β -catenin/Tcf-mediated transcription pathway caused by mutations of the APC or β -catenin gene plays an important role in colon carcinogenesis. In the present study, β -catenin expression was localized in the membranes at the cell-cell border (Figure 2: 1A-1C) in colonic mucosa. This finding is consistent with the previous studies by Iwamoto et al. (2000) and Ochiai et al. (2003). This finding highlighted the roles of molecule in the maintenance of normal function and properties of colon cancer.

In our study, the adenoma and adenocarcinoma samples, β -catenin immunoreactivity was found to be variable with heterogenous staining patterns in the cell membrane and cytoplasm. These are consistent with the published data by Kohno et al. (2005) and Ochiai et al. (2003). The expression of β -catenin was also found in the cell membrane and cytoplasm of adenoma and carcinoma with the β -catenin intensity weaker in adenoma compared to adenocarcinoma samples. The results however, are contradictory with the study by Kohno et al. (2005) who found strong β -catenin expression in the nucleus and cytoplasm of adenocarcinoma samples. β -catenin binds to the cytoplasmic tail of alpha-catenin (α -catenin) and E-cadherin indirectly with cytoskeleton. Therefore, β -catenin is mainly localized to the adherens junction at the cell-to-cell plasma membrane. β -catenin may dissociate from α -catenin and E-cadherin and enters into the cytoplasm as free unbound β -catenin. This explains the presence of β -catenin in the cytoplasm. The less intense stain of β -catenin in the normal colonic tissue compared to colorectal carcinoma shows that the β -catenin level in the tumor is much greater than in normal colonic tissue. Since β -catenin is an important Wnt signaling pathway activator, its level in the normal tissue is constantly regulated by APC, axin, glycogen and glycogen kinase synthase-3 β complex that targets it for degradation via ubiquitination in the cytoplasm. As a result, no β -catenin enters the nucleus. This explains the absence of β -catenin in the nucleus. The ability of PA to retard the movement of β -catenin from the cytoplasm to the nucleus when the β -catenin or APC genes are mutated or Wnt signaling pathway is activated may explain this provision. For the confirmation of β -catenin expression, we have included the colorectal carcinoma (human sample) (processed under similar condition with the rats specimens) as positive control and data demonstrated strong nuclear and cytoplasmic β -catenin immunoreactivity. Therefore, we have verified the β -catenin staining from our rat specimens. Earlier study by Latifah et al., (2010) also showed that β -catenin expression in AOM-induced colon cancer rats was reduced by treatment with germinated brown rice (GBR). They also suggested that beside fiber and ferulic acid, phytic acid which is found in GBR could also be involved in the reduction of total number of ACF in these rats.

The role of COX-2 as an enhancer of carcinogenesis in many organs including the colon is receiving increasing attention. COX-2 is related to the formation of carcinogens, tumor promotion, apoptosis inhibition, angiogenesis development, and metastatic process (Meric et al., 2006). Up-regulation of expression of COX-2 has been associated with human intestinal inflammation, colorectal cancer (Wang and Dubois, 2010) and worse survival among CRC patients (Ogino et al., 2008). Therefore, COX-2 expression may be used to monitor the process of carcinogenesis, and the suppression of COX-2 expression can become a target for cancer chemoprevention (Singh et al., 1997). In the present study, COX-2 expression was also observed in normal colonic mucosa. This result is in agreement with the reports by Kohno et al. (2005) that showed the expression of COX-2 even in normal colon mucosa but in contrast with Shao

et al. (1999). Results from our study however, shows that the expression of COX-2 protein in colon tumors was higher than in normal colonic mucosa, suggesting that this differential expression of COX-2 protein is closely related to events leading to the development of colon tumors. This is in agreement with a study by Suzuki et al. (2007) who reported increasingly high levels of AOM-induced COX-2 in the colonic mucosa. It is also well accepted that COX-2 protein levels are elevated in carcinogen-induced rodent colon tumors (DuBois et al., 2001) and in most colorectal cancer patients (Lim et al., 2007).

The potential mechanism that may contribute in suppressing the expression of COX-2 is due to the ability of PA inhibition of rate-limiting step to catalyze the conversion of arachidonic acid into prostaglandins. The cyclooxygenase (COX), also known as prostaglandin-enderoperoxide synthases, transform arachidonic acid into prostaglandin G₂ (PGG₂), by acting both as dioxygenase and a peroxidase (Tuynman et al., 2001). Therefore, by inhibiting the step to catalyze the conversion of arachidonic acid into prostaglandins, the process such as inflammatory reactions, gastro-intestinal protection, haemostasis and renal haemodynamics can also be inhibited (Merici et al., 2006). Earlier studies also demonstrated that fermented brown rice and rice bran (Phuttaphadong et al., 2010), rice bran (Li et al., 2010) and GBR (Latifah et al., 2010) inhibit colon carcinogenesis and COX-2 expression. Phytic acid which was found to be 9.5-14.5% in rice bran (Jarriwala, 2001) was suggested could partly be involved in these effects (Latifah et al., 2010).

Increased proliferation of colon epithelial cell, characterized as hyperplasia can be detected with Ki-67 proliferation marker (Kikuchi et al., 1997). Therefore, we evaluated whether phytic acid (PA) suppresses the amount of Ki-67 positive cells in colon tissue. Our result of Ki-67 clearly showed a significant increase in proliferative activity of tumor group either in adenoma or adenocarcinoma compared to the groups that received PA. Reduced tumor incidence is generally associated with a decrease in cellular proliferation and/or increases in apoptosis (Barnes et al., 1996). Therefore, the ability of PA to inhibit cell proliferation activity and induce apoptosis in colorectal mucosa is in accordance with this finding and further supports earlier report by Shamsuddin et al. (1988). However, the difference did not have a statistical significance ($p > 0.05$) within each group of treatment. The difference between the two percentage of PA (0.2% w/v and 0.5% w/v) used as a treatment may have not demonstrated the difference to be seen. Therefore, increasing percentage of PA may possibly assist in observing the difference between different groups of treatment.

The results of our study demonstrated the relationship between expression of β -catenin, COX-2 and Ki-67. A good correlation was noted with degree of reduction in cell proliferation. We have found that Ki-67 score has a significantly positive relationship with total COX-2 expression. Study by Sheehan et al. (2004) which is consistent with our finding demonstrated that COX-2 expression may contribute to colonic tumorigenesis by promoting cell growth, new vessel formation, invasiveness

and, in particular, metastatic potential. Ki-67 also has positive linear relationship with β -catenin expression. The relationships however, was not statistically significant ($p > 0.05$). This shows that an increase in proliferative activity is concurrent with increased total β -catenin expression. Thus, it suggested disturbance in the balance between the rate of proliferation and cell death results of tumor cell growth. Tetsu and McCormick (1999) reported that in several types of human cancer, mutation in the β -catenin or APC gene causes accelerated tumor cell proliferation and tumor progression through the transcriptional activation of target genes such as cyclin D1, with resulting cytoplasmic/nuclear accumulation of β -catenin (Morin et al., 1997). We have found that the expression pattern between β -catenin and COX-2 are well correlated. Our finding was in line with the study by Takahashi et al. (2000), which reported frequent mutation, and an altered cellular localization of β -catenin in rat colon adenocarcinomas induced by AOM, along with up-regulation of COX-2. The increased level of COX-2 proved evidence of an involvement of the Wnt-APC- β -catenin/Tcf pathway in COX-2 expression as reported by Tanaka et al. (2001).

In conclusion, Phytic acid (PA) was significantly suppressed β -catenin activity, COX-2 expression and Ki-67 proliferative activity in AOM-induced rats and thus showed their potential use as an alternative treatment for colon cancer. Further investigation is needed to ascertain the exact mechanism of action involved.

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