# THE EFFECT OF CELLULOSE ON APOPTOSIS OF COLON EPITHELIAL CELLS OF BALB/C MICE THAT INDUCED BY 9,10-DIMETHYL-1,2-BENZ(A)ANTHRACENE

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## ABSTRACT

*Incidence and death caused by cancer remains high. Epidemiological studies show that dietary fiber, including cellulose, may reduce the incidence of colon cancer. Cellulose is one of dietary fiber that can not be digested by digestive enzyme but can be fermented by colon microflora to produce butyrate, a short chain fatty acid (SCFA). Butyrate inhibits histone deacetylase (HDAC) enzyme and the process of apoptosis. However, the effect of cellulose on apoptosis of colon epithelial cells are not known. The aim of this study was to prove increasing the number apoptotic colon epithelial cells of BALB/C mice that were fed with cellulose and then were exposed to 9,10-dimethyl-1,2-benz(a) anthracene (DMBA).The design of this experiment was posttest only control group design. The male BALB/C mice of 12 weeks old with body weight of 30 - 40 grams were used in this experiment and were given feed containing 0% or 5% cellulose. Two weeks after cellulose administration the mice were exposed to DMBA and sacrificed 5 days later. The colon was removed and processed in the paraffin block, then was stained with TUNNEL (terminal deoxyuridine nucleotide end labeling) assay technique with apoptag detection kit. The data were analyzed by T-test. Results showed that there was difference ($p < 0.05$) between the number of apoptotic colon epithelial cells in mice fed with cellulose and those that were not fed with cellulose. The difference was marked by increasing the number of apoptotic colon epithelial cells in mice fed with cellulose. The conclusion of this experiment is that cellulose can increase the number of apoptotic colon epithelial cells of BALB/C mice that are exposed by DMBA. The effect of cellulose on colon carcinogenesis presents as the increasing number of apoptotic colon epithelial cells that are exposed by DMBA.*

***Keywords**: cellulose, DMBA, apoptosis*

## INTRODUCTION

Incidence and death resulted from colorectal cancer in the United States remains high. The incidence of colorectal cancer in 1998 was 1,228,660 cases, and almost 50% finally ended in death (Cotran, 1999). Epidemiological studies showed that individuals and populations that consume high dietary fiber and vegetables a lower risk against colon cancer (Cummings, 1992; Muir, 1998). McIntyre (1993) reported that the incidence of colon cancer induced by chemical carcinogen reduces in rats fed with high dietary fiber. The reduction of cancer incidence induced by chemical carcinogens is related to cellular capability in cell cycle arrest, providing sufficient time to repair DNA damage. If the damage cannot be repaired, the cell will be apoptosis, preventing it to become a cancerous cell (Cotran, 1999). Cellulose is one type of dietary fibers that have capability in reducing incidence of colon cancer due to chemical carcinogens (Hardman, 1997). However, its effect of cellulose in apoptosis remains unclear.

Cellulose is one type of dietary fibers that have capability in reducing incidence of colon cancer due to chemical carcinogens (Hardman, 1997). Cellulose can not be digested by digestive enzyme but can be fermented by colon microflora, and one of the products of the fermentation is butyric acid (Brody, 1994; Mandal,1998). It is the butyric acid that plays a role in the reduction of colon cancer incidence by activating the transcription of gene p21 that results in cell cycle arrest (Archer,1999; Archer, 1998). In addition, butyric acid may also enhance Bax expression and reduce Bcl2 expression (Guiliano,1999; Ruemmele, 1999). Cell cycle arrest will give time to repair DNA damage and if the repair fails, cell became apoptosis. Enhancing Bax expression induce apoptosis. If apoptosis fails too, DNA cells became permanent damage (DNA mutated) and carcinogenesis will be begin. Apoptosis in this conditions plays the importance role to prevent carcinogenesis at initiation stage.

According to that explanations, this paper discusses whether cellulose may increase the number of apoptotic colon epithelial cells in male BALB/C mice exposed to 9,10-dimethyl-1,2-benz(a)anthracene (DMBA). The general objective of this study was to disclose the protective effect of cellulose against carcinogenesis in colon epithelial cells of mice due to the exposure to carcinogenic chemicals, while the particular objective was to demonstrate increasing number of apoptotic colon epithelial cells exposed to DMBA in mice fed with cellulose. The benefits of this study were:

1. To provide scientific information for the development of science in nutrition, health, and medicine on the protective effect of cellulose

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against carcinogenesis in colon epithelial cells due to chemical carcinogens exposure.
2. To provide scientific information for the community on materials that can be employed to prevent colon carcinogenesis, i.e., by using cellulose as dietary fiber source to prevent colon carcinogenesis in order to reduce incidence of cancer and to increase prosperity of community.

## MATERIALS AND METHOD

### Materials

Experimental animals used in this study were male BALB/C strain mice aged 12 weeks with bodyweight of 25 - 35 grams. Materials used were cellulose, pellet feeds, water, 9,10-dimethyl-1,2-benz(a)anthracene (DMBA) from Sigma, apoptag detection kit from Intergen, and materials used for tissue processing and staining. Equipments used were mice cage made from plastic and covered with wire netting, completed with plates for food and bottles for drink, sonde, a set of surgical tools, microtome, waterbath, object glass, cover glass, labels, microscope, and pencils.

### Methods

Cellulose was mixed in the feed and given to the experimental animals in pellet. During the adaptation period, control and treatment group was given with feed containing cellulose 0%. During the study, control group was given with feed containing cellulose 0% and treatment group was given with feed containing cellulose 5%. DMBA was given using abdominal sonde with dose 10 mg/100 g BW. Before the study was commenced, cage and DMBA were prepared. Cage was washed and then disinfected one week before use to make it sterile. Feed was mixed in the Feeds Laboratory, Airlangga University School of Veterinary Medicine. There were two types of feeds given to the experimental animals, i.e., feed with 0% and 5% cellulose. DMBA used was 9,10-dimethyl-1,2-benz(a)anthracene from sigma. DMBA was solved using olium olivarum to facilitate per oral administration.

Mice were taken from the population, and randomly divided into two groups receiving the following treatments:
1. Control: During the study, mice were given with feed containing cellulose of 0%, and at day 15 they were exposed to DMBA with dose 10 mg per 100 g BW.
2. Treatment: Mice in this group were given with feed containing cellulose of 5%, and at day 15 they were also exposed to DMBA with dose 10 mg per 100 g BW.

Mice were adapted to the conditions of the study one week before the research being to do. During the adaptation, they were given with pellet containing only 0% cellulose. Feed and drink were given ad libitum during the study.

Treatment was started after the adaptation. Control group was given with feeds containing 0% cellulose, while treatment group was given with those containing 5% cellulose. Mice were scaled to determine DMBA dose and then exposed to DMBA using abdominal sonde at day 15. DMBA was exposed at day 15 after cellulose feeding with the hope that it may provide an optimum effect to colon epithelial cells. The procedure was similar to that of Boffa (1992) who exposed carcinogenic agents two weeks after the administration of treatment feeds. DMBA was used as an exposing agent to colon epithelial cells in this study as it was a potential chemical carcinogenic agent for all tissues in human and animals.

Mice were sacrificed 5 days after DMBA exposure (day 20 in the research process) using single fixative method. Mice were subsequently dissection and their colon was fixation in buffer formalin solution of 10%. The colon was then processed to create preparations in paraffin blocs and being stained using HE staining and TUNEL (Terminal Deoxyuridine Nukleotida End Labeling) technique by means of apoptag detection kit. According to this technique, apoptotic cells are cells that indicate fragmentation DNA with the free 3-OH termini. The free 3-OH DNA fragment termini was label to digoxigenin-nucleotide triphosphat. Finally with adding chomogenin substrate, the nuclei of apoptotic cells became brown and the nuclei of non apoptotic cells became green counter stain.

The staining slide were observed using light microscope with magnification of 400x, and the number of apoptotic colon epithelial cells per 100 cells was counted. Each slide was observed in 4 observational fields clockwise at 3, 6, 9, and 12 o'clock. Each observation field was observed and counted at two fields clockwise at 6 and 12 o'clock using grateculae. The results were averaged. Data collected were analyzed by means of T test.

## RESULTS

Examination of the number apoptotic colon epithelial cells in mice was undertaken by the way observing and counting apoptotic cells per observation field. In each

sample, observation and counting was undertaken to apoptotic cells in four observation fields clockwise at 3, 6, 9, and 12 o'clock. Total apoptotic cells in one observation field were observed and counted at two fields clockwise at 12 and 6 o'clock using grateculae in magnification of 400 x. The number of apoptotic cells was counted per 100 cells.

Average results of observation and counting of the number apoptotic colon epithelial cells in male BALB/C mice are presented in table 1 and figure 1. Table 2 presents results of mice colon staining using apoptag detection kit without TDT enzyme and digoxygenin-labeled nucleotide in experimental animals fed with cellulose and exposed to DMBA. Results of this staining were used as staining control. All colon epithelial cells were green, indicating no binding between digoxygenin-labeled nucleotide and 3-OH terminal of DNA fragmentation, so that brownish dye that appeared in the staining using apoptag detection kit indicated binding between digoxygenin-labeled nucleotide and 3-OH end of DNA fragmentation.

Results of Hematoxylin Eosin (HE) in colon epithelial cells in male BALB/C mice fed with cellulose and exposed to DMBA are presented in figure 3. The staining was done to demonstrate that by means of HE staining, stained nuclei were in larger number than those stained with apoptag detection kit. Brownish dye appeared in apoptag detection kit staining resulted from apoptosis, while non-apoptotic cells were unstained. Using HE, the nuclei were stained in purplish blue.

Results of mice colon staining using apoptag detection kit in mice fed without cellulose and exposed to DMBA are presented in figure 4, and those from mice fed with cellulose and also exposed to DMBA are presented in figure 5.

Collected data were analyzed by means of T test to identify the difference of the number apoptotic colon epithelial cells in male BALB/C mice fed with cellulose and those fed without cellulose. T-test results of the number apoptotic colon epithelial cells in mice fed with cellulose were significantly different ($p < 0.05$) from those fed without cellulose.

Table 1. The average of number apoptotic colon epithelial cells in male BALB/C mice per 100 cells

| Treatment | Mean | Standard Deviation |
|---|---|---|
| Control | 2.104 | 1.993 |
| Treatment | 36.75 | 6.896 |

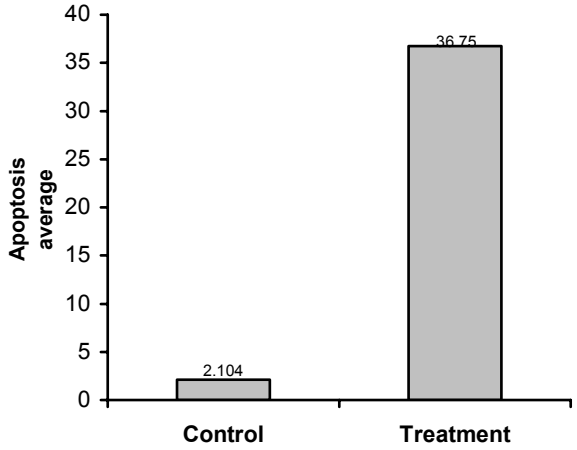


Figure 1. Bar diagram of the number apoptotic colon epithelial cells in mice fed with cellulose and exposed to DMBA.

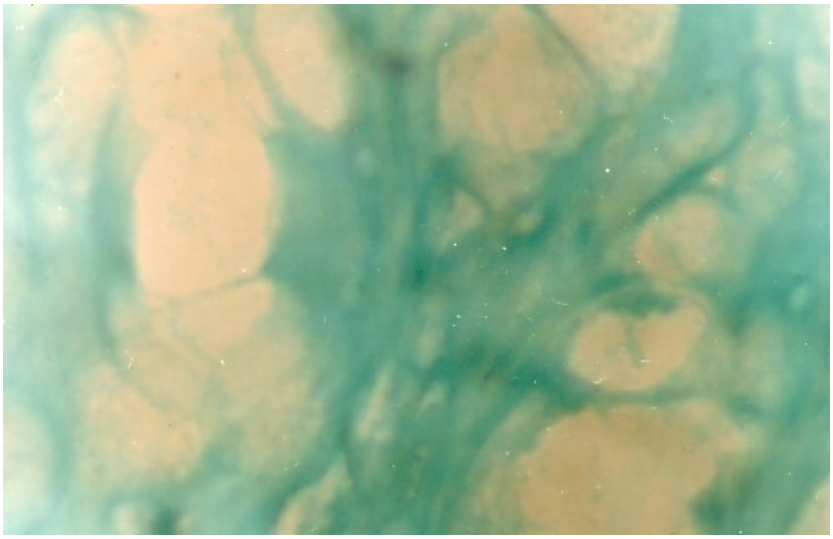

Figure 2. Results of apoptag detection kit staining without Tdt and digoxygenin-labeled nucleotide in colon epithelial cells of male BALB/C mice fed with cellulose and exposed to DMBA, in magnification 400 x.
Note : Green dye indicates no binding between 3-OH from DNA fragments and digoxygenin-labeled nucleotide.

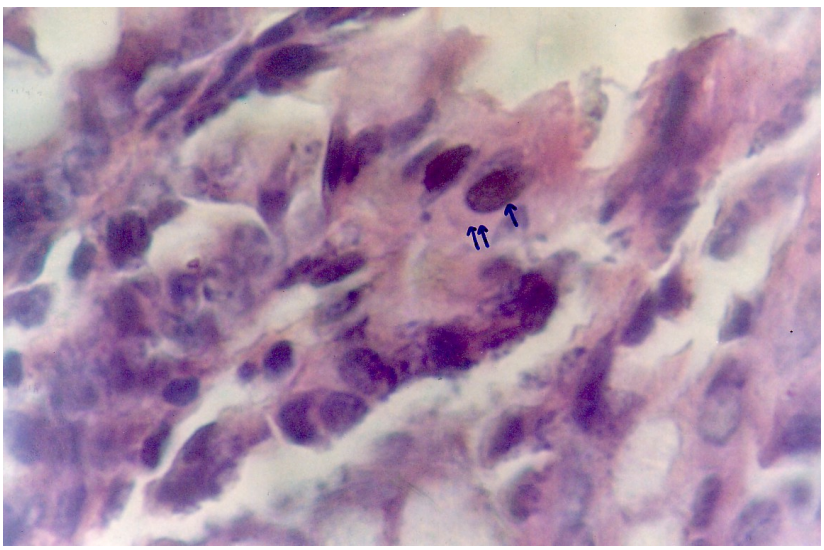

Figure 3. Results of Hematoxylin Eosin (HE) staining in colon epithelial cells in male BALB/C mice fed with cellulose and exposed to DMBA with magnification 400 x.
Note: nuclei (one arrows) : purple, cytoplasm (two arrows) : eosin.

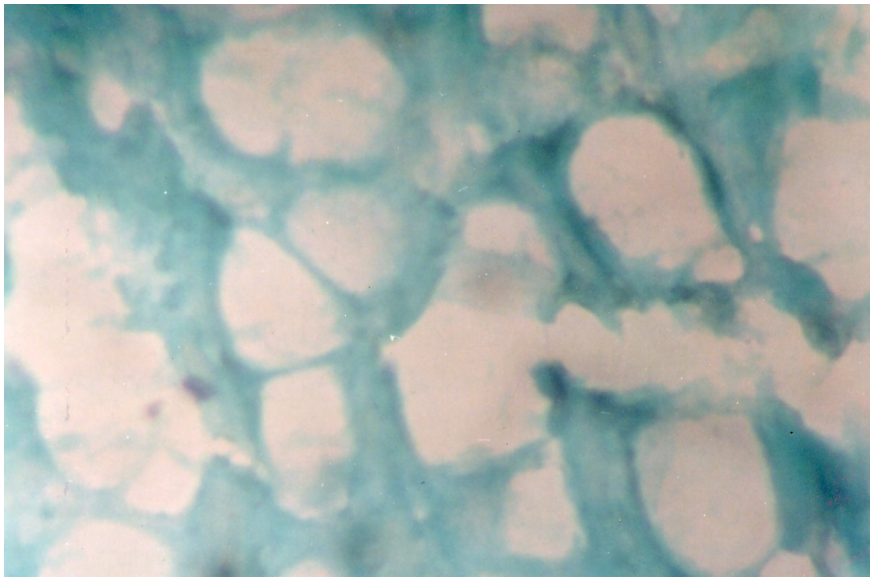

Figure 4. Results of staining with apoptag detection kit in colon epithelial cells in male BALB/C mice fed without cellulose and exposed to DMBA with magnification 400 x.
Note: brownish dye: apoptotic cells, green: non-apoptotic cells.

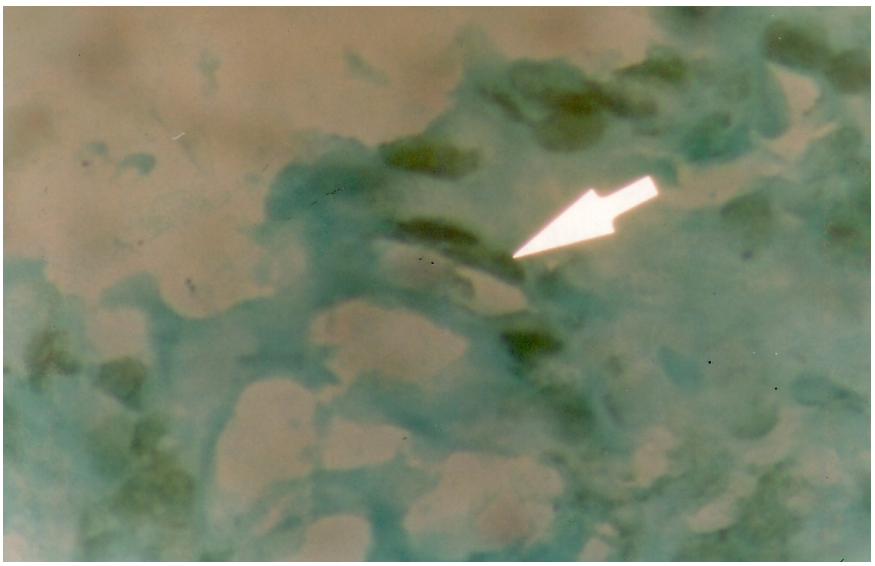

Figure 5. Results of staining with apoptag detection kit in colon epithelial cells in male BALB/C mice fed with cellulose and exposed to DMBA with magnification 400 x.
Note: brownish dye: apoptotic cells (pointed), green: non-apoptotic cells.

## DISCUSSION

One characteristic in the apoptosis is the DNA fragmentation (Cotran, 1999). DNA fragmentation in apoptosis can be detected by means of TUNEL (Terminal Deoxyuridine Nucleotide End Labeling) assay, which is the basis of apoptag detection kit. The principle in TUNEL assay was to label 3-OH terminal from DNA fragments with digoxygenin nucleotide triphosphat enzymatically. This reaction was catalyzed by TDT (terminal deoxynucleotidyl transferase) enzyme. Random nucleotide labeling was intended to trigger optimum binding between digoxygenin and antidigoxygenin (Anonimous, 1998).

Results of TUNEL assay with apoptag detection kit can be seen in figures 2, 4, and 5. Staining with apoptag detection kit without TDT enzyme and digoxygenin-labeled nucleotide was used as control staining. Staining in such method was aimed to prevent binding between 3-OH terminal from DNA fragment with triphosphat nucleotide, so that binding between digoxygenin and antidigoxygenin peroxidase conjugate can be prevented, and eventually cells were stained only with fast green (counter stain). Results of such staining in mice fed with cellulose and exposed to DMBA showed that all cells were stained green, which was the fast green counter stain, and no cells stained brown.

Staining with apoptag detection kit using TDT enzyme and digoxygenin labeled nucleotide in mice colon that similar to used for control staining, indicated that there were cells that stained green, and there were cells that also stained brown. It demonstrate that control staining, there was no binding between at 3-OH terminal of DNA fragment and digoxygenin-labeled triphosphate nucleotide and no binding between digoxygenin and antidigoxygenin peroxidase conjugate, so that with the addition of DAB (diamino benzidine), which was a chromogenin substrate, cells were not stained brown, and they only absorbed green dye from counter stain. Staining with TDT enzyme and digoxygenin-labeled nucleotide resulted in binding between 3-OH terminal of DNA fragment and digoxygenin-labeled triphosphate nucleotide and binding between digoxygenin and antidigoxigenin peroxidase, so that the addition of chromogenin substrate might render conjugate cells with 3-OH terminal from DNA fragment to have brown dye, while other cells became green. The brownish dye resulted from binding between 3-OH terminal from DNA fragment and digoxygenin-labeled nucleotide, digoxygenin and antidigoxygenin peroxidase conjugate, and between peroxidase and DAB substrate.

Based on the results of HE staining (figure 3) in the same mice colon as those used for control staining, it was found that stained nuclei were in larger number than those stained with apoptag detection kit. It indicated that brownish dye appeared in cells containing 3-OH terminal from DNA fragment in staining with apoptag detection kit resulted from binding between 3-OH terminal with active substrate from apoptag detection kit and chromogenin. Other cells that contained no 3-OH terminal from DNA fragment were not stained with apoptag detection kit, but by means of HE, the nuclei appeared to be purplish blue.

Dimethylbenz(a)anthracene (DMBA) is a chemical carcinogen that used in this study. DMBA belonged to polycyclic aromatic hydrocarbon (PAH), which is procarcinogenic (Cotran, 1999). DMBA is a result of tobacco burning, particularly in cigarette smoking, product of animal fat during the process of meat or fish roasting or smoking (Devita, 1993). Human beings unconsciously often make contact with DMBA, and even consume foods that contain DMBA, although DMBA is a carcinogenic chemical that potentially triggers carcinogenesis in almost all tissues in human and animal. Therefore it is importance to prevent Carcinogenesis due to DMBA.

DMBA exposure might reduce apoptosis in colon epithelial cells in mice, and the reduction was different from that in mice not exposed to DMBA. The apoptotic reduction occurred at day 5 after DMBA exposure (Mastutik, 2001). Compher (1999) showed that at day 5 after exposure, cell had entered the tumor initiation stage. This stage is important in the carcinogenesis process since mutation or permanent damage in the DNA has occurred. If exposed to materials that serve as promoter, a cell with damaged DNA may become cancerous. Cell has a defense mechanism to prevent the initiation stage, i.e., by DNA repair, and if it fails, it will apoptosis

Apoptosis is important to prevent carcinogenesis. Apoptosis is cell death that occurs in several conditions, i.e., during cellular growth, as homeostatic mechanism such as in immunologic reaction when cells were damaged by adverse agents, and in aging (Cotran, 1999). Apoptosis found in this study was that occurred when cells were damaged by chemical agents. The agents were the chemical carcinogen DMBA. DMBA in those cells were metabolized by cytochrome enzyme P-450 to become 8,9-diol-10,11-epoxide, that had a highly reactive electron and capability to bind with DNA, particularly in adenine and guanine base (Yang, 1988; Wijnhoven, 1998). The binding triggered defense mechanism to eliminate the injury by activating DNA repair and apoptosis.

Failure in DNA repair and apoptosis renders the injury to become permanent and enhances carcinogenesis. Permanent injury in cellular DNA may activate oncogen, changing apoptosis-regulating genes, and inactivating cancer suppressor gene. All of these factors may cause change in gene products and the loss of gene regulating products (Cotran, 1999). Change in apopotosis-regulating gene may eliminate cell population controller in the tissue. Cancer suppressor gene basically inhibits cancer growth, so that inactivation of the gene may also eliminate cancer growth inhibitor. Action disorder in these three important genes that control normal cell growth renders the cell to become an abnormal clone, to experience progression, and finally to grow as cancerous cell.

Epidemiological studies showed that dietary fiber is an important factor in reducing the incidence of colon cancer. However, its mechanism remains unknown. Cotran (1999) suggested that one of molecular factor in the emergence of cancer is the damage in apoptosis-regulating gene. The disorder in apoptotic function causes homeostatic mechanism disorder in controlling cell population in the tissue and in the process of mutated-cell elimination. Mutation in the protooncogene, cancer suppressor gene and apoptotic gene may lead to carcinogenesis. Therefore, as long as mutation or DNA damage can be repaired or apoptosized, carcinogenesis can be prevented. In contrast, if DNA repair and apoptosis fails, carcinogenesis may ensue.

Results of this study showed that the number of apoptotic colon epithelial cells in mice fed with cellulose and exposed to DMBA was significantly different from mice fed without cellulose. The difference was marked by the increase of apoptotic colon epithelial cells in cellulose-fed mice. As discussed above, apoptosis is important in the process of carcinogenesis, as the increase of apoptosis may inhibit carcinogenesis. In fact, this study showed that feeds containing cellulose might increase apoptosis in colon epithelial cells in mice that exposed to DMBA. The DMBA exposure, a chemical carcinogen that potentially triggers carcinogenesis, should have reduced the number of apoptotic colon epithelial cells. Instead, DMBA exposure in mice fed with cellulose increased the number of apoptosis. It indicated that cellulose addition to animal's feeds might reduce the number of mutated cells by enhancing apoptosis so that it finally might prevent carcinogenesis.

Results of this study confirmed those of Ranhotra (1999) and Hardman (1997). Ranhotra (1999) reported that the addition of cellulose in mice feeds could reduce the incidence of colon cancer induced by chemical carcinogens. Hardman (1997) suggested that there was a reduction of colon cancer incidence in patients consuming cellulose compared to those who did not consume cellulose.

The reduction mechanism of colon cancer incidence may result from cellulose capability in affecting apoptosis. Cellulose is a dietary fiber, a component of cell wall in plants, which is indigestible by human digestive enzymes (Murray, 1998). Cellulose is one of the most commonly found dietary fibers in nature. It is composed from a number of glucose units that form a linear polymer chain. Cellulose is digestible by bacterial enzyme, cellulase, in large intestine to produce energy, hydrogen, carbon dioxide, methane, and short chain fatty acid (SCFA). SCFA consists of acetic, propionic, and butyric acids. Butyrate is utilized by colon cells, while acetate and propionate travel in the circulation and utilized by other organs (Sanford, 1992; Brody, 1994). It is butyrate that plays an important role in preventing or reducing the incidence of colon cancer. Diet containing a large amount of fibers produces much more butyrate, with the results that it may eventually provide protective effect against colon carcinogenesis (McIntyre, 1993; Boffa, 1992).

Butyrate is known to be able to trigger histone acetylation, particularly in the hyperacetylation H3 and H4 through the inhibitor histone deacethylase (HDAC). Histone acetylation level depends on the activity of two enzyme families, HDAC and histone acetyltransferase (HAT). HDAC inhibition activates HAT, so that inhibition of HDAC can activated HAT and it cause histone hyperacetylation. Butyric may inhibit HDAC via protein serine threonin phosphatase (Archer, 1999).

Butyrate inhibits the activity of enzyme HDAC in locus gene p21 in mice through the element cis that located 1.4 kb from the transcription start site that results in histone hyperacetylation and the activation transcription of gene p21. The increase of p21 expression leads to cell cycle arrest by blocking cyclin dependent kinase (CDK) and the activity of proliferating cell nuclear antigen (PCNA) (Archer, 1998; Archer, 1999). It provides a sufficient time for the cell to repair injured DNA. If the repair fails, the cell may commit apoptosis. Failed DNA repair and failed apoptosis may results in carcinogenesis (Cotran, 1999).

Giuliano (1999) and Ruemmele (1999) suggested that butyric acid might increase Bax expression and reduce Bcl2. Butyric acid also stimulates the change of procaspase-3 to become caspase-3. Chai (2000) reported that butyrate triggers apoptosis in two ways, i.e., by inhibiting HDAC and synthesizing and activating DEVD (Asp-Glu-Val-Asp) Caspase (Caspase-3).

DEVD caspase activating may trigger PARP (Poly ADP-Ribose Polymerase) breakage, marking an onset of DNA fragmentation.

Compared to control, the addition of cellulose to the feed of male BALB/C mice exposed to DMBA could increase the number of apoptotic colon epithelial cells. The number of apoptotic colon epithelial cells in control was smaller than that in treatment since the control of DNA repair mechanism and apoptosis failed, so that DNA injury became permanent and colon carcinogenesis started to ensue. In treatment group, the number of apoptotic colon epithelial cells in control was larger, indicating that the increase of apoptotic mechanism was able to prevent carcinogenesis, since cells with injured DNA had been apoptosized.

The addition of cellulose to the feed of male BALB/C mice could increase the number of apoptotic colon epithelial cells, resulting in the prevention of permanent injury in the DNA due to the DMBA. Thus, the protective effect of cellulose against carcinogenesis due to the exposure to DMBA is by increasing the number of apoptotic colon epithelial cells that exposed to DMBA, resulting in the prevention of permanent DNA injury and inhibition of carcinogenesis.

## CONCLUSION

The conclusion of this experiment is that cellulose can increase the number apoptotic colon epithelial cells of BALB/C mice that are exposed by DMBA. The effect of cellulose on colon carcinogenesis presents as the increasing of apoptotic colon epithelial cells that are exposed by DMBA.

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