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Effect of Dietary Fatty Acids on Colon Tumorigenesis Induced by Methyl Nitrosourea in Rats

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To study the effect of dietary fatty acid on the colon tumorigenesis induced by methyl nitrosourea in rats, male SD rats were fed five semi-synthetic diets composed of different proportions of beef tallow, soybean oil, alkana oil, corn oil and fish oil for 180 days. The experimental groups were injected with a solution of methyl nitrosourea in phosphate buffer intraperitoneally once a week for six weeks. The control groups were injected with phosphate buffer solution only. The incidence of colon cancer, the avergage volume of the tumors, proliferation cell nuclear antigen, cell kinetics, membrane lipid fluidity, alkaline phosphatase activities and the content of prostaglandin E_2 in colon mucosa and the fatty acid of testis pad fat were measured at the end of the experiment. The results showed that the incidence of colon cancer and the average volume of tumors in animals fed with diets composed mainly of beef tallow, soybean oil or alkana oil were significantly higher than those that were fed fish oil. The most effective anticancer diet in our study contained saturated fatty acid, monounsaturated fatty acid and polyunsaturated fatty acid of fish oil in the proportion of 13.9%, 16.4% and 68.8% respectively. Inhibition of colon tumorigenesis appeared to be related to the regulation of membrane lipid fluidity, and a decrease in the proliferation of cell nuclear antigen in colon cells. In addition, a decrease was noted in the number of cells in S phase and alkaline phosphatase activity, along with inhibition of arachidonic acid products and a corresponding decrease in the amount of prostaglandin E_2.

INTRODUCTION

Dietary factors may play an important role in the development of human cancers. Several epidemiological studies and animal models have shown that the source and amount of dietary fat could influence the risk of colon cancer (Vogel and McPherson, 1989). One earlier cohort study on colorectal cancers in Japanese men living in Hawaii (Stemmerman *et al.*, 1984) showed a decreasing risk with higher intakes of total fat. The low colon cancer rate in Alaskan residents who habitually consumed fish products rich in *n*-3 polyunsaturated fatty acids (*n*-3 PUFAs) suggested that fish oil may be a protective factor in colon carcino-

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Abbreviations: MNU, methyl nitrosourea; PBS, phosphate buffer solution; i.p., intraperitoneal; PCNA, proliferation cell nuclear antigen; ALP, alkaline phosphatase; PGE_2, prostaglandin E_2; EPA, eicosapentaenoic acid (C20:5, *n*-3); SFA, saturated fatty acid; DHA, docosahexaenoic acid (C22:6, *n*-3); PUFA, polyunsaturated fatty acid; LA, linoleic acid (C18:2, *n*-6); α-LNA, α-linolenic acid (C18:3, *n*-3); β-LNA, β-linolenic acid (C18:3, *n*-6); MUFA, monounsaturated fatty acid; PG, prostaglandin; AA, arachidonic acid.

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genesis (Blot *et al*., 1975). On the other hand, some studies found that the most commonly used vegetable oils, such as corn oil and safflower oil containing large amounts of *n*-6 PUFA, may promote colon carcinogenesis in rats (Reddy and Maeura, 1984; Takeshita *et al*., 1997). One of the reasons is that the *n*-6 series fatty acids could alter the kinetics of colon cells, and increase proliferation of cell nuclear antigen (PCNA) (Thornton and MacDonald, 1994). Diets rich in fish oil tend to have an inhibitory effect on carcinogenesis (Reddy and Sugie, 1988). Fish oil is rich in *n*-3 PUFAs, such as eicosapentaenoic acid (EPA, C20:5, *n*-3) and docosahexaenoic acid (DHA, C22:6, *n*-3). Either fish oil or DHA or EPA separately could inhibit colon cancer formation induced by chemical carcinogens compared with linoleic acid (LA, C18:2, *n*-6) (Reddy, Burill and Rigotty, 1991; Takahashi *et al*., 1993; Oshima *et al*., 1995). Fish oil seems to influence early stages of experimental colon carcinogenesis, by decreasing cell proliferation and dysplasia in azoxymethane treated rats (Deschner *et al*., 1990). These studies support the hypothesis that the fatty acid composition of the diet is more important than the overall fat content for colon cancer risk.

Howe (Howe, 1997) analyzed 13 case-control studies of colorectal cancer involving 5,287 cases and 10,478 controls from various populations with differing cancer rates and dietary practices. There was no evidence of any increased colorectal cancer risk with higher dietary fat after adjustment for total energy intake. And there were no statistically significant associations for any type of fat in sub-group analyses by sex, age, or anatomic location of the cancer. Thus this report failed to provide evidence for the association of colorectal cancer with dietary fat. A large number of studies using a number of rodent strains and various carcinogens also showed that expression of intestinal tumor genes is enhanced as the quantity of dietary fat is increased. All the above data indicates that fatty acid composition may influence the risk of colorectal cancer.

Alkana oil is rich in both LA and α-linolenic acid (α-LNA, C18:3, *n*-3) and β-LNA (C18:3, *n*-6). Some experimental studies showed that α-LNA inhibited the growth of cancer cell lines (Salerno and Smith, 1991). This effect of α-LNA on colon carcinogenesis induced by chemical carcinogens in rats was not found. It is interesting to note that experimental studies using animal models support the hypothesis that fatty acid composition of dietary fat is one of the determinant factors in colon carcinogenesis.

In this study, different approaches were adpoted to assess the possible role of dietary fatty acids, from beef tallow, corn oil, alkana oil and fish oil, in preventing tumorigenesis in colon cancers induced by methyl nitrosourea (MNU) in rats.

MATERIALS AND METHODS

Animals

90 male Sprague-Dawley rats (body weight, 123 ± 11g) were housed individually in stainless steel cages in an environmentally controlled room with 12h light/dark cycles. They were randomly divided into 5 groups. Each group was randomly divided into a control group of 8 animals and an experimental group of 10 animals according to their weight. The animals in the experimental groups were injected with methyl nitrosourea (MNU) intraperitoneally (i.p.) at a dose of 30mg/kg body weight, once a week for 6 weeks. The animals in the control group were injected with an equal volume of phosphate buffer solution (PBS). Food intake and body weights were recorded daily.

Diets

Nutritionally adequate diets were prepared based on the AIN-76 rat diet (1977). The fat content of each diet was the same (15%, w/w) (Table 1). Each diet contained 2% corn oil in order to prevent a deficiency of essential fatty acids. The fatty acid profiles of beef tallow, corn oil, alkana oil and fish oil were determined by gas chromatography. The oils were then mixed according to a designed fatty acid composition ratio. The fatty acid composition in each group's diet are shown in Table 2. All experimental semi-synthetic diets were prepared in our laboratory 3 times weekly and stored at 4℃ in air-tight plastic containers filled with nitrogen. The malondialdehyde content of the freshly prepared experimental diets and that of stored diets were analyzed routinely using the thiobarbituric acid method

TABLE 1

The Main Composition of the Diets

Component	%
Casein	20.0
Cornstarch	30.0
Sucrose	30.0
DL-methionine	0.3
AIN mineral mix.[a]	3.5
AIN Vitamin mix.[b]	1.0
Choline bitartrate	0.2
Fat	15.0

[a] Composition of mineral mixture (g/kg mix.): $CaHPO_4$ 500.0; NaCl 74.0; $K_2C_6H_5O_7 \cdot H_2O$ 220.0; K_2SO_4 52.0; MgO 24.0; $MnCO_3$ 3.5; Ferric citrate 6.0; $ZnCO_3$ 1.6; $CuCO_3$ 0.3; KIO_3 0.01; $NaSeO_3 \cdot 5H_2O$ 0.01; $CrK(SO_4)_2 \cdot 12H_2O$ 0.55; Sucrose to make 1000g

[b] Composition of vitamin mixture (mg/kg mix.): Thiamin HCl 600; Riboflavin 600; Pyridoxine. HCl 700; Nicotinic acid 3,000; D-calcicium pantothenate 1,600; Folic acid 200; D-biotin 20; Vitamin E 5,000IU; Vitamin A 400,000IU; Vitamin B_{12} 1mg; Vitamin D_3 2.5; Vitamin K 5.0; sucrose to make 1,000g.

TABLE 2

The Fatty Acids Composition of Diets (%)

Group	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3	C18:4	C20:5
1	20.9	3.4	26.9	30.5	12.9	5.7	ND	ND
2	11.9	ND	5.0	26.3	56.0	7.0	ND	ND
3	5.8	ND	2.2	14.2	27.2	38.5	11.2	ND
4	11.7	0.3	2.3	16.1	20.1	8.9	ND	15.4
5	10.0	1.0	8.9	21.0	26.4	15.2	2.8	5.0

Group	C22:6	SFA	MUFA	PUFA	*n*-6/*n*-3	M/S	P/S
1	ND	47.8	33.9	18.6	ND	0.709	0.389
2	ND	16.9	26.3	63.0	ND	1.556	3.728
3	ND	8.0	14.2	76.9	4.53	1.775	9.613
4	24.4	13.9	16.4	68.8	0.73	1.180	4.950
5	7.9	18.9	22.0	57.3	1.95	1.164	3.032

Note. SFA: saturated fatty acid; MUFA: monounsaturated fatty acid; ND: not detected.

(Yu and Liu, 1989). The range of malondialdehyde which formed in all diets stored for 48h was from 2.5 to 5.6 nmol/g, and the values in different dietary oils were not significantly different. Vitamins A and E were added at a dose of 2.5 mg/kg and 0.1 mg/kg to different oils in order to make the concentration of these vitamins uniform.

Experimental Procedure

After having been fed the diets for 2 days, the animals in the experimental groups were injected with methyl nitrosourea (MNU) intraperitoneally (i.p.) at a dose of 30mg/kg body weight, once a week for 6 weeks, and the control group was injected with equal volume of phosphate buffer solution (PBS). Animals were sacrificed under diethyl ether anesthesia 182 days later. The colons were taken, and the number of tumors was counted and the volume of each tumor was measured. The lower 2cm of the large intestine was placed into 10% neutral-buffered formalin, run up through the alcohol series, and embedded in paraffin. 3 μm sections of the tissue were obtained and mounted. Slides were developed and stained with hematoxylin and eosin, and examined with a light microscope. The number of cells per crypt column was counted by two people individually.

Determination of the PCNA Expression in Nuclei of Cells and Propidium Iodine Labeled Cells in Different Phases

According to the method of Thornton and MacDonald (1994), nuclear suspensions were incubated with anti-PCNA antibody and then were stained with propidium iodine and analyzed by flow cytometry (Becton Dickinson). At least 1×10^4 nuclei of cells was analyzed for each animal.

Determination of the Membrane Lipid Fluidity of Colon Mucosa Cells

Distal and proximal colon was washed with ice-cold saline. The mucosa was scraped with a slide into a plate containing PBS (pH 7.4), centrifuged with 1500 rpm/min for 7 min, and the precipitate was added to pepsin 0.4mg/L in 0.01 mol/L HCl. The mixture was incubated at 37℃, vibrated for 13 min, and then centrifuged at 1500 rpm/min for 10 min. The precipitate was diluted with 2.5% bovine serum albumin in PBS, and 10^9 cells solution was put into 4 ml of 2×10^{-6} mol/L diphenyl-hexatriene and incubated at 25℃ for 30 min. The polarization was determined by fluorescence-polarization (Beccerica, Piergiacomi and Guratola, 1988; Zhou, Wang and Chen, 1996).

Determination of Testis Pad's Fatting Acid Composition

The fatty acid composition of fat in the testis pad was determined by gas-liquid chromatography-mass spectrometry.

Determination of the Activity of Alkaline Phosphatase (ALP) and the Content of Prostaglandin E_2 (PGE_2) in the Colon Mucosa

The activity of alkaline phosphatase (ALP) and the content of prostaglandin E_2 (PGE_2) in the colon mucosa homogenate were determined according to the instruction of the ALP kit

which was purchased from the Zhongsheng Biological Company. PGE_2 kit was purchaced from the Beijing Greatwall Biological Company.

Statistical Methods

Data were analyzed by the significant difference test. Data are expressed as $\bar{x} \pm s$. Analysis of variance (ANOVA) was used to determine differences among the groups. Differences are regarded as significant when the P value was less than 0.05. The difference of tumor incidence among different dietary groups was tested by χ^2 test.

RESULTS

Food Intake and Body Weight

The food intake and energy intake was not different among the five groups (Fig. 1). However, 98 days later, the average body weight of the 4th group was significant less than that of the others (Fig. 2).

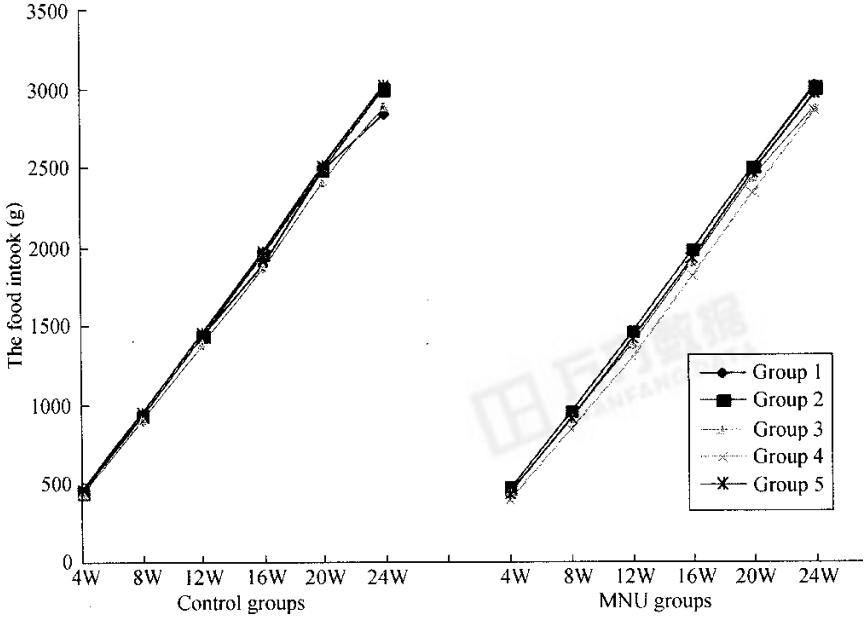

FIG. 1. Food intake by rats in the control and MNU groups during the experimental period. The MNU groups received methyl nitrosourea, the control groups, physiological saline. The diets of groups 1-5 had different fatty acid composition, for details see Table 2.

The Number of Cells per Crypt Column and Colon Carcinoma, the Average Volume of Tumors in Colon

The number of cells per crypt column in the distal colon in the 3rd group was significantly higher than that of the 4th group, and not different in the proximal colon (Fig. 3). Most of the tumors induced by MNU were highly differentiated adenocarcinomas. The number of colon carcinomas per rat was significantly lower in the 4th group than in the others. The average volume of tumors was slightly smaller in the 4th and 5th groups than that of the others. The number of tumors in each rat was significantly lower in the 4th group compared to other groups (Table 3).

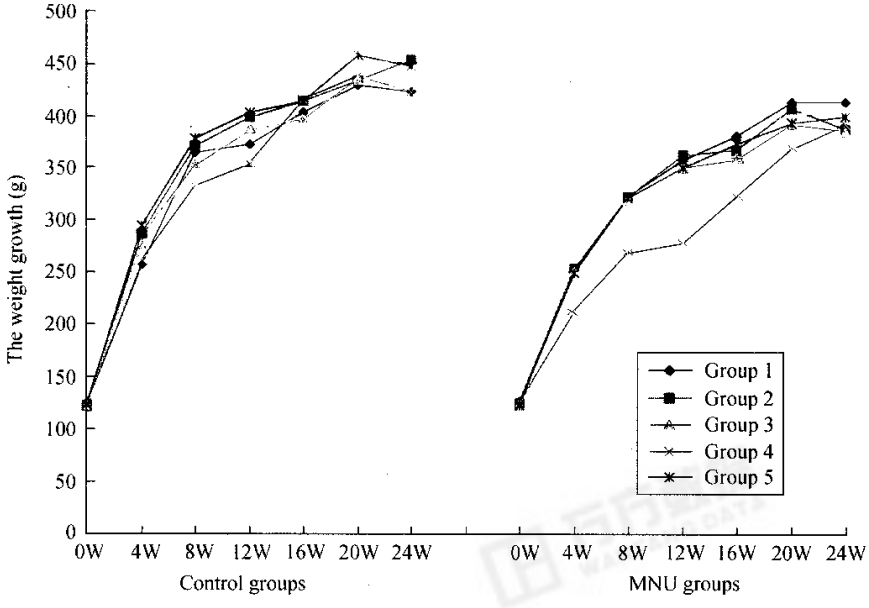

FIG. 2. The weight growth of rats in the control and MNU groups during the experimental period. The MNU groups received methyl nitrosourea, the control groups, physiological saline. The diet of groups 1-5 had different fatty acid composition. For details see Table 2.

Effect on PCNA Expression and the Percentage of Colon Cells in Phases of the Cell Cycle

PCNA expression in the nuclei of cells was the highest in rats of the 3rd group, and lower in both the 4th and the 1st group, as was the percentage of colon cells in the S phase of the cell cycle as determined by propidium iodine staining. There were no differences of the percentage of colon cells in G_1 and $G_2 + M$ phases of the cell cycle among other groups (Table 4).

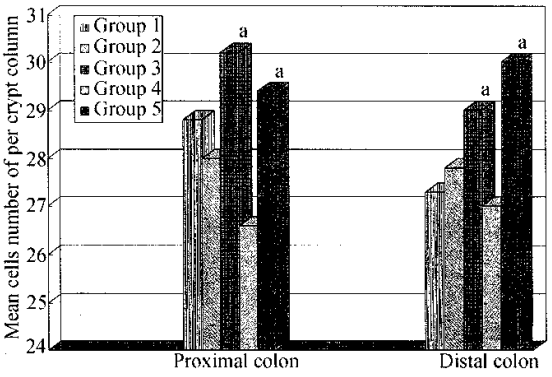

FIG. 3. Mean cells number of per crypt column determined by histology in colon sections. Colon samples were collected from 5 rats in each of the diet groups and 10 crypt columns were analyzed per region per rat. Numbers with different superscripts are statistically different at $P < 0.05$ as determined by f significant difference.

TABLE 3

The Number of Colon Carcinoma per Rat, the Average Volume of Tumors and the Number of Rats Bearing Tumors Among Different Group

Group	Number of Colon Carcinoma per Rat	Average Volume of Tumors (mm^3/tumor)	Number of Rats Bearing Tumors	Total Number of Rats
1	2.34 ± 1.37^a	133 ± 213^a	6^a	10
2	2.98 ± 1.56^a	156 ± 321^a	6^a	10
3	3.57 ± 1.92^a	239 ± 345^a	5^a	10
4	0.52 ± 0.23^b	65 ± 84^b	1^b	10
5	1.87 ± 1.12^a	79 ± 94^b	4	10

Note. Values in a column not sharing a common superscript are significantly different ($P < 0.05$).

TABLE 4

PCNA Expression in Nuclei of Cells and the Percentage of Colon Cells in Each Phase of Cell Cycle

Group	PCNA-Positive-Staining Cells (%)	Percentage of Colon Cells		
		G_1	S	$G_2 + M$
1	17.1 ± 1.2^a	89	3.4^a	5.4
2	24.7 ± 4.9^b	88	4.8^b	5.0
3	34.5 ± 11.8^c	87	6.6^c	4.7
4	18.9 ± 3.6^a	92	3.2^a	5.2

Note. Values in a column not sharing a common superscript are significantly different ($P < 0.05$).

Effect on the Activity of ALP, Membrane Lipid Fluidity and PGE_2 in Colon Mucosa

The polarization in the colon cells of the 4th group was the lowest, thus the fluidity of the cells was the best. The content of PGE_2 was significantly lower in the 4th group than that in the 1st and the 2nd group. The activity of ALP was the lowest in the 4th group (Figs. 4-6).

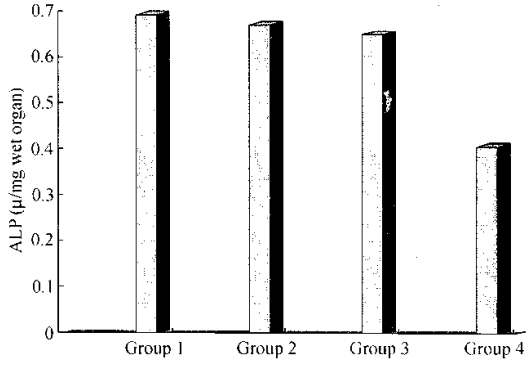

FIG. 4. The activity of ALP in colon mucosa.

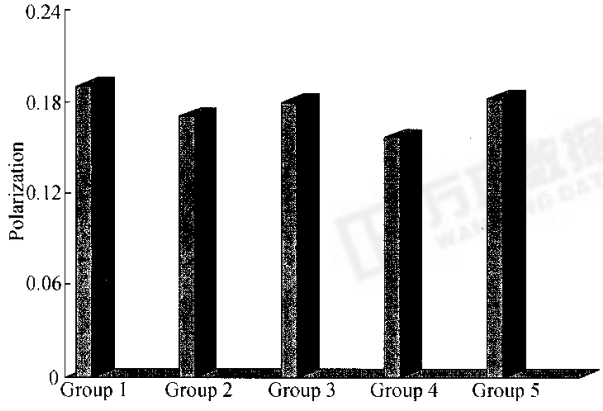

FIG. 5. The polarization of fluidity in colon mucosa.

Effect on the Fatty Acid Composition in Testis Pad Fat

Among the five groups, the percent of C20:4 was lower in the 4th group than that in the 3rd group, but higher than that in the 1st and 2nd groups. The percent ages of C20:5, C22:6 and PUFA in the 4th group were the highest among the five groups (Table 5).

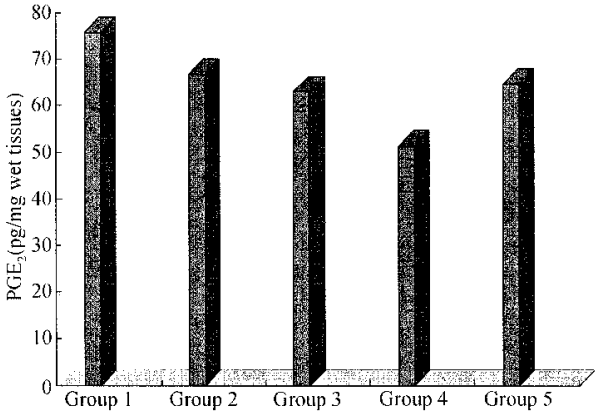

FIG. 6. The content of PGE_2 in colon mucosa.

TABLE 5

Some Fatty Acids Composition in Testis Pad Fat (%)

Group	C18:2	C18:3	C20:4	C20:5	C22:6
1	14.8 ± 1.70^a	0.2 ± 0.09^a	2.4 ± 1.03^a	0.4 ± 0.22^a	3.0 ± 0.40^a
2	22.9 ± 1.57^b	0.3 ± 0.12^a	5.9 ± 4.98^b	0.4 ± 0.21^a	2.5 ± 0.42^a
3	17.3 ± 2.62^c	2.1 ± 0.44^b	15.0 ± 3.33^b	2.3 ± 0.40^b	2.2 ± 0.51^a
4	19.7 ± 2.42^c	0.1 ± 0.01^a	7.9 ± 1.61^c	4.5 ± 0.24^c	13.4 ± 1.02^b
5	19.9 ± 1.11^c	0.3 ± 0.13^a	10.3 ± 3.05	3.7 ± 1.14^c	10.4 ± 1.53^c

Group	SFA	MUFA	PUFA	*n*-6:*n*-3
1	34.4 ± 3.25	27.5 ± 3.98^a	31.0 ± 2.49^a	18.71
2	30.9 ± 2.14	24.0 ± 1.63^a	41.9 ± 3.27	13.00
3	28.9 ± 1.67	13.8 ± 1.12^b	39.1 ± 2.18^b	7.00
4	32.2 ± 2.32	13.5 ± 1.53^b	46.3 ± 3.16^c	1.56
5	34.1 ± 1.89	12.3 ± 1.06^b	45.3 ± 1.98^c	2.14

Note. Values in a column not sharing a common superscript are significantly different ($P < 0.05$).

DISCUSSION

During the experimental period, important factors such as dietary energy, antioxidants and peroxidants that may influence the development of tumor was controlled. The growth of transplanted tumors has been shown to be reduced by limiting the intake of carbohydrate or fat (Pariza, 1987; Reddy, 1992). In this experiment the rats in different groups were given the same amount of food, and so the food intakes of these groups were not significantly different. After vitamins A and E were determined in each diet by HPLC, other antioxidants

such as BHA and BHT were added to all groups at the same level to retard the breakdown of PUFA. Therefore, the difference in the incidence of tumors among the groups was only caused by differences in the fatty acids composition in each diet.

The present study shows that the number of tumors generated in the 4th group was the least and the tumor size was the smallest among the five groups. This finding indicates that the diet containing 15% fat, with a fatty acid composition of 13.9% SFA, 16.4% MUFA and 68.8% PUFA may suppress the formation and growth of colon tumors induced by MNU in SD male rats. In the 3rd group with greater proportion of α and γ-LNA, there was still a high tumor incidence. This result need to be further investigated.

The mechanisms responsible for observed tumor suppression in the 4th group are not known. Expression of PCNA is an endogenous marker of cell proliferation (Mathews *et al.*, 1984). PCNA is an auxiliary protein of DNA polymerase-δ and plays a role in the initiation of cell proliferation (Ravo *et al.*, 1987). PCNA is not expressed by cells in Go, but is expressed as the cell progresses from late G_1 into early S phase. Two PCNA subpopulations exist within the cell nucleus; about 30% of the PCNA is tightly associated with DNA replication sites while the rest diffuses into the nucleoplasm (Ravo *et al.*, 1987; Kitamoto *et al.*, 1993). Increased PCNA expression has been observed in rats during progression from a preneoplastic phase to a neoplastic one (Risio, 1992; Yamada *et al.*, 1992). Thornton (1994) using a normal animal model, found that an increase in the number of cells in G_1 and showed a reduction of the number of cells in S was associated with increased dietary fat. They thought that diets high in *n*-6 PUFA showed more risk for colon cancer than diets high in SFA. Table 3 shows a great difference in PCNA expression among the groups induced by different chemical carcinogens. The PCNA expression in the third group was the highest. The percentage of colon cells in S phase correlated with the cells marked by anti-PCNA. This indicates that PCNA is closely associated with the S phases. In our studies, PCNA is the last in the 4th group, which indicates that the PCNA expression in colon mucous cells may be effected by dietary fatty acid composition. *n*-3 PUFA in the 4th group was about 6 times higher than that in the 3rd group. It could be inferred that at the same amount of fat, a higher percentage of *n*-3 series fatty acids, corresponds to less PCNA/cycle express, on which reduces the activity of δ-DNA polymerase and delays the progression from preneoplasia to neoplasia. PCNA expression in the 1st group was also less, which might be due to the fact that the diet in this group contained more SFA (47%). The SFA has less ability to induce PCNA expression than does PUFA (Thornton and MacDonald, 1994).

Appropriate membrane lipid fluidity is important for the physiological function of a cell. Membrane fluidity is affected by properties of the membrane lipid. Membranes rich in PUFA have greater fluidity. The polarization is one of the parameters used to examine membrane fluidity. A higher value of polarization of a cell, the lower lipid fluidity of cell membranes. Small changes in membrane lipid fluidity occuring in micro-environments may be responsible for several functional alterations (Spector and Burns, 1987).

In this study, polarization of cells in one group increased as the amount of saturated fatty acid in their diets was increased (Table 3). The polarization in the 1st group was the highest, because of the highest ratio of saturated fat, whereas the polarization in the 4th group was the lowest, because of the highest ratio of polyunsaturated fat. The diet of the 4th group had a slightly higher ratio of saturated fatty acid than that of 3rd group. With a higher ratio of *n*-3/*n*-6, the fluidity was the highest in the 4th group. The reason for the lowest tumor incidence in this group was not known. However, it is likely that with more PUFA, more free radicals were produced, which would be harmful to normal cells and beneficial to the tumor cells.

There is evidence that drugs which inhibit PG synthesis can prevent or reduce development of chemically induced tumors. Treatment of carcinogen-induced animals with PG inhibitors reduced the incidence of colon cancer (Reddy, Murayama and Kelloff, 1987). PGs are primarily derived from arachidonic acid (AA) which originates from LA. It was shown that AA in the testis pad was lower in the 1st and 4th groups than that in the 2nd, 3rd and 5th groups (Table 4). *n*-3 fatty acids can inhibit the metabolism of AA. The diet in the 4th group contained the most omega-3 fatty acids among the five groups.

Activity of ALP was associated with the increased proliferation of several cell types. In this study, the results showed that the activity of ALP was the highest in the 3rd group which contained the lowest total SFA and highest PUFA (mainly of the *n*-6 series). This finding coincided with the number of tumor-bearing rats.

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