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Effects of Sardine Oil Feeding and Vitamin E Supplement on the Preneoplastic Hepatic Lesion and Cholesterol Metabolism in Hepatocarcinogenesis of Rats

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Abstract

This study was done to investigate effects of n-3 fatty acids and vitamin E supplement on the preneoplastic hepatic enzyme altered foci and cholesterol metabolism in experimental hepatocarcinogenesis system. Weaning male Sprague-Dawley rats were fed the diet containing either 15% corn oil(CO) or sardine oil(SO) with or without vitamin E 800IU supplementation for 12 weeks. After two weeks of feeding, rats were intraperitoneally injected with a single dose of diethylnitrosamine(DEN: 200mg/kg, BW). At the 4th week, rats were given the diet containing 0.02% acetylaminofluorene(AAF) for next 4 weeks. At the 6th week, 0.05% phenobarbital was incorporated into diet for 6 weeks. At the end of 12th week, rats were sacrificed and hepatic glutathione S-transferase placental form positive(GST-P^+) foci and serum and liver cholesterol levels were determined. GST-P^+ formation was significantly decreased by SO feeding when compared with CO feeding but it tended to be enhanced by vitamin E supplementation. Histopathological changes were similar to patterns of GST-P^+ formation in almost all dietary groups. Serum and hepatic cholesterol levels of SO fed groups were significantly lower than those of CO fed groups. Carcinogen treatments significantly increased serum and liver cholesterol levels in CO fed groups but not in SO fed groups. Correlation data showed a positive correlation($r=0.83$, $p<0.01$) between serum cholesterol level and GST-P^+ foci area. These results indicate that sardine oil as a n-3 fatty acid source may have a reducing effect on rat hepatocarcinogenesis by the alteration of cholesterol metabolism.

Key words: GST-P^- foci, sardine oil, vitamin E, cholesterol

INTRODUCTION

Both epidemiological evidence(1) and experimental studies(2) have shown that the dietary fatty acid composition as well as the amount of dietary fat play an important modulatory role in cancer incidence. Particularly, many animal studies have reported that corn oil rich in n-6 fatty acids increase cancer induction while fish oil rich in n-3 fatty acids suppress it(3,4). But most of investigations have been mainly performed in the experimental models of breast and colon cancer. There are few reports to investigate the effect of n-3 fatty acids on hepatocarcinogenesis although some studies examined the effect of the amount of dietary fat and polyunsaturated n-6 fatty acids(5).

Since early epidemiological observations(6) indicated that individuals with low concentration of serum cholesterol were at increased risk of cancer, many studies have focused on elucidating the relationship between serum cholesterol and cancer incidence. But overall informations so far have shown inconsistent relationships between serum cholesterol levels and both total and site-specific cancer risks(7). Therefore, the suggestion that a low plasma cholesterol is an important risk factor for cancer have not confirmed. Particularly, Hsing et al.(8) reported that liver cancer mortality in China was higher in the population with high plasma cholesterol level.

An important feature of malignant transformation is loss of the cholesterol feedback inhibition that regulates cholesterol synthesis(9). Cancer cell seem to require an increase in the concentrations of cholesterol and cholesterol precursors. There are some evidence that that lowering the plasma cholesterol level and intervening in the mevalonate pathway with HMG-CoA reductase inhibitors decreases tumor growth *in vivo* and cell cul-

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ture experiments, respectively(10). Buchwald(11) suggests the hypothesis that cholesterol inhibition can inhibit tumor cell growth and possibly can prevent carcinogenesis.

Vitamin E as an antioxidant has been shown to be important in the prevention of cancer(12). However, Ura et al.(13) reported that the time of vitamin E supplementation is also important to modulate carcinogenesis.

The quantification of enzyme-altered foci is frequently employed as a measure of multistage hepatic carcinogenesis. It is generally believed that measurement of GST-P^+ foci is regarded as a useful marker for preneoplastic hepatic lesions and mechanistically related to hepatic tumorigenesis(14). GST-P^+ single cells and mini-foci are formed in carcinogen-treated rat livers very early prior to the formation of nodules(15). It is hardly detectable in normal rat liver, while highly expressed in preneoplastic liver lesions.

The present study was conducted to investigate effects of n-3 fatty acids and vitamin E supplement on the preneoplastic hepatic enzyme altered foci and cholesterol metabolism in experimental rat hepatocarcinogenesis system.

MATERIALS AND METHODS

Experimental diets and design

Eighty male Sprague-Dawley rats weighing 80~90g fed 8 different experimental diets. All experimental rats were kept in wire bottomed cage in a room at 20±2°C with 07:00-19:00 light-dark cycle, and water ad libitum. The diet compositions were based on AIN-76 diet : corn starch 55.2%, α-cellulose 5.0%, casein 20.0%, DL-methionine 0.3%, oil 15.0%, salt mixture 3.5%, vitamin mixture 1.0%(vitamin E supplement 0.08%). All diets were composed of 15% of either corn oil or sardine oil with or without dl-α-tocopherol acetate(800IU/kg diet) supplementation. The basal diet for each oil groups contains 50IU of dl-α-tocopherol acetate per kg diet if the vitamin E content of dietary oil itself is not considered.

As shown in experimental protocol(Fig. 1), after two weeks of feeding, rats were intraperitoneally injected with a single dose of DEN(200mg/kg, BW). At the 4th week, rats were given 0.02% 2-AAF in diet for next 4 weeks. At the 6th week, 0.05% phenobarbital was incorporated into diet for 6 weeks.

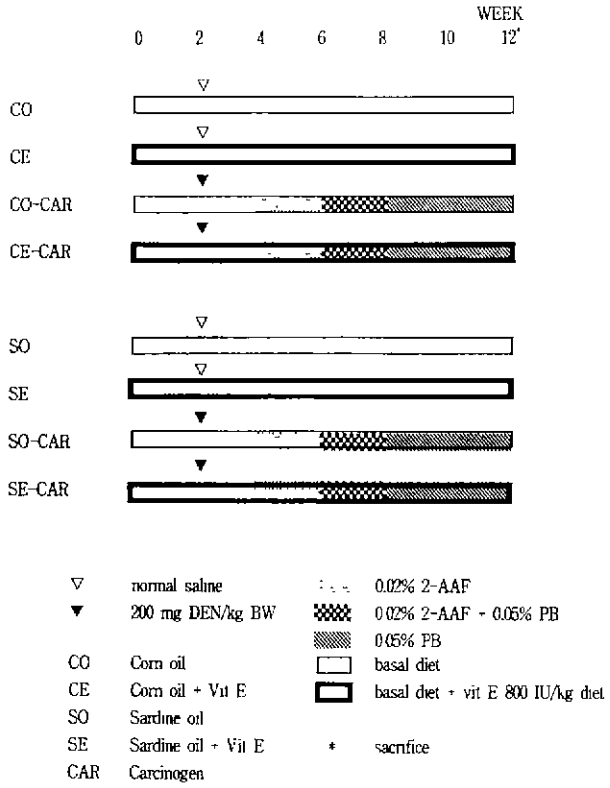

Fig. 1. Experimental design.

Histopathological examination & quantification of GST-P^+ foci

At the end of 12th week, rats were sacrificed and the liver was excised in 1~2mm thickness with a razor blade immediately upon killing. Two slices were fixed in ice-acetone, processed for embedding in paraffin and stained with hematoxylin and eosin for histopathological examination. The scores of liver cirrhosis, necrosis, fatty change, septal fibrosis, dysplastic cell, septal implamm-ation, clear cell foci, basophilic foci and eosinophilic foci were measured by optical microscope. Subsequent immunohistochemical examination of GST-P^+ foci was done by ABC method(Vectastain ABC kit)(16). Rabbit anti GST-P^+ was provided by Korea Cancer Center Hospital. The number, area and mean diameter of GST-P^+ foci greater than 0.2mm diameter were measured with a video image processor and expressed as number/cm^2, areas(mm^2)/cm^2 or mean diameters(mm).

Serum and hepatic cholesterol

Serum cholesterol levels were determined by enzymatic method(17) using cholesterol kit. Liver lipids were extracted by Bligh and Dyer method(18). Liver cholesterol

was then determined in aliquots of the lipid extracts by the enzymatic method of Allain et al.(17) as modified by Sale et al.(19).

Statistics

The significance of differences between means was analyzed by analysis of variance(ANOVA) and Duncan multiple range test using Statistical Analysis System (SAS) program. Values of $p<0.05$ were accepted as being statistically significant. Correlation between cholesterol level and foci formation was carried out by pearson correlation analysis

RESULTS

Body weight and liver weight

Body weights of animals were not significantly affected by carcinogen treatment. But liver weights were significantly increased. Therefore, the liver weight per body weight was significantly increased by the carcinogen treatment(Table 1). The morphological change of liver by appearance was observed in carcinogen treated groups. The severity was greater in carcinogen treated groups with vitamin E supplement.

GST-P$^+$ foci and histopathologic finding

The induction of GST-P$^+$ hepatic foci in rats treated with carcinogens was significantly increased while non-carcinogen treated rats showed almost no foci(Fig. 2).

Table 1. Effects of sardine oil feeding and vitamin E supplementation on body and liver weights in carcinogen treated rats

Experimental group	Body weight(g)	Liver weight(g)	Liver weight/Body weight(%)
CO	326.6±13.9^{ab}	8.67±0.40^{a}	2.67±0.09^{a}
CO-CAR	331.0± 9.5^{ab}	17.79±1.80^{b}	5.33±0.40^{b}
CE	354.4±18.9^{b}	9.80±0.57^{a}	2.27±0.06^{a}
CE-CAR	324.7± 9.9^{ab}	20.36±1.99^{b}	6.22±0.48^{c}
SO	309.7±15.5^{a}	9.51±0.64^{a}	3.07±0.11^{a}
SO-CAR	338.8±20.5^{ab}	17.43±1.32^{b}	5.13±0.16^{b}
SE	321.0±20.1^{ab}	9.96±0.55^{a}	3.14±0.23^{a}
SE-CAR	334.2±18.5^{ab}	18.35±1.24^{b}	5.48±0.15^{b}

Values are mean±S.E.
Means with different superscrips within same column are significantly different at $p<0.05$ by Duncan's multiple range test

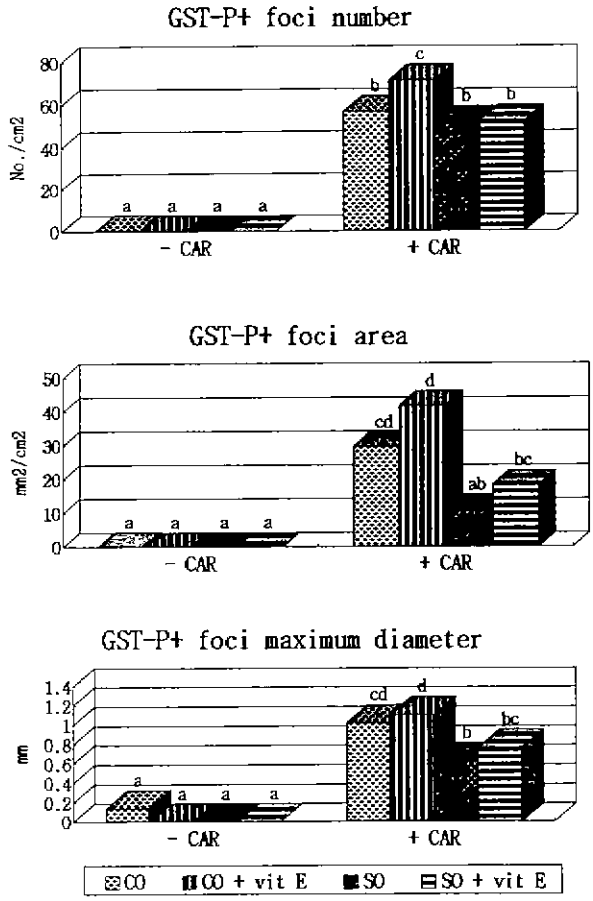

Fig. 2. Effects of sardine oil feeding and vitamin E supplement on the number, area and maximum diameter of hepatic GST-P$^+$ foci in carcinogen treated rats.

Table 2. Histopathologic finding in carcinogen treated rats with sardine oil feeding and vitamine E supplementation

	L	N	F	SF	D	I	CF	BF	EF
CO	0	0	0	0	0	0	0	0	0
CE	0	0	0	0	0	0.1	0	0	0
SO	0	0	0	0	0	0	0	0	0
SE	0	0	0	0	0	0	0	0	0
CO-CAR	0	0	1.6	1	0	1.2	0.6	0.6	0.4
CE-CAR	1.4	0.8	1.8	1	0.8	2	0.8	0	0
SO-CAR	0	0	1	1.2	0	1.2	0.4	0.2	0.6
SE-CAR	0	0	1.4	0.4	0.2	1.8	0.4	0	0.4

L: liver cirrhosis, N: necrosis, F: fatty change, SF: septal fibrosis, D: displastic cells, I: septal inflammation, CF: clear cell foci, BF: basophilic foci, EF: eosinophilic foci
The severity of hepatic lesion for each parameter is scored as follows
If hepatic all lesion is below 1/3: 1point, 1/3~2/3: 2point, above 2/3: 3point

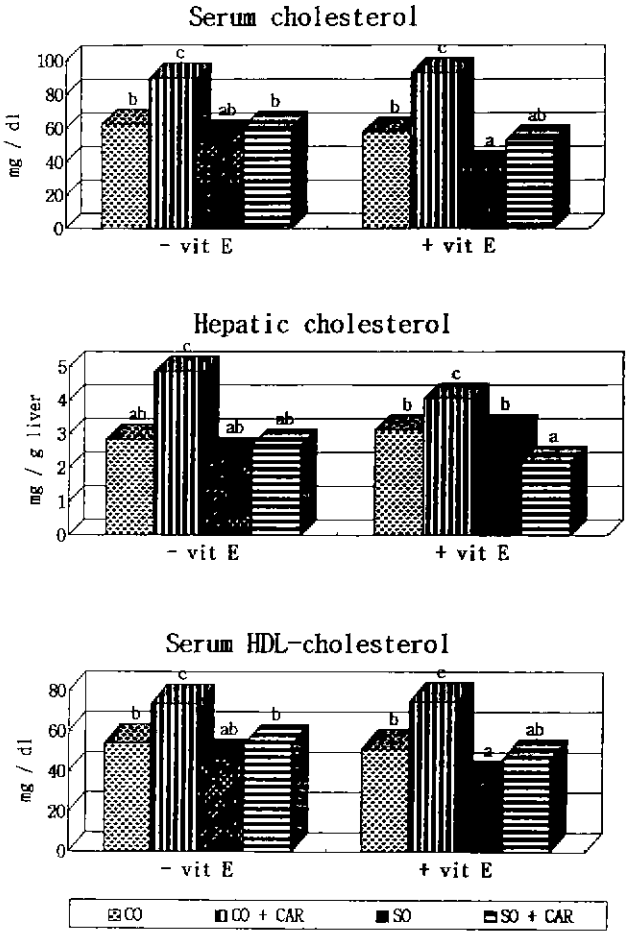

Fig. 3. Effects of sardine oil feeding and vitamin E supplement on the serum and hepatic cholesterol and HDL-cholesterol levels in carcinogen treated rats.

The number of GST-P^+ foci was not significantly different but area and mean diameter of GST-P^+ foci were significantly decreased by SO feeding compared with CO feeding. The vitamin E supplementation tended to enhance GST-P^+ foci formation but overall effect was not significant. Similar to the patterns of GST-P^+ foci data, the score for histopathological changes seemed to be greater in CO groups than SO groups(Table 2).

Serum and liver cholesterol

As shown in Fig. 3, serum and hepatic cholesterol levels of SO groups were significantly lower than those of CO groups. Particularly, carcinogen treatment significantly increased serum cholesterol level in CO groups while not significantly increased in SO groups. Similar to serum cholesterol level, hepatic cholesterol level and serum HDL-cholesterol level were also significantly increased by carcinogen treatment in CO groups but not in SO groups. Vitamin E supplementation did not affect serum and hepatic cholesterol levels.

DISCUSSION

Increasing dietary fat content enhances the development of chemically and aflatoxin B1(AFB)-induced neoplasms and r-glutamyl transpeptidase(GGT)-positive foci in rats(20). Furthermore diet rich in polyunsaturated fatty acids enhance hepatocarcinogenesis(21). Recent studies suggest that the enhancement of hepatocarcinogenesis by dietary fat is primarily due to an effect on initiation and that polyunsaturated fats have a greater effect than do saturated fats(5).

The end-point lesion used in the present study was GST-P^+ hepatic foci, regarded as the most useful marker for the preneoplastic hepatic lesion(15,22). The dietary modulating effect was compared by scoring the number, area/cm^2 and mean maximum diameter (mm) of GST-P^+ foci introduced in the liver of carcinogen treated rats. The induction of GST-P^+ hepatic foci in rats treated with carcinogens was significantly decreased by SO feeding compared with CO feeding. Similar to the patterns of GST-P^+ foci data, overall scores of histopathological and morphological changes were lower in SO groups than in CO groups. William(23) reported that these morphological changes at rat liver by carcinogen treatment could develop preneoplastic lesions. Therefore our results might indicate that n-3 fatty acid in sardin oil could reduce hepatocarcinogenesis.

In this study, vitamin E supplementation tended to enhance hepatocarcinogenesis rather than to inhibit it. This result was similar to the data of Kim and Choi(24) who examined the effect of vitamin E supplement on GST$^+$-foci formation in rats fed either corn oil or perillar oil. Ura et al.(13) investigated the effect of dietary vitamin E on the steps of hepatocarcinogenesis, the induction and growth of γ-glutamytranspeptidase-positive foci in the liver of rats treated with DEN. Their results suggest that vitamin E prevent the very early events during hepatocarcinogenesis, the induction of phenotypically altered foci, but could no longer affect the later stage, the evolution stage of foci into persistent nodules. Therefore the effect of vitamin E supplement on carcinogenesis seems to depend on the time and period of supplementation. Since we supplemented dl-alpha-tocopherol acetate throughout the experiment period, our result might be different from that of Ura et al.(13).

It has been suggested that low total serum cholesterol level may be an important risk factor for cancer incidence in early epidemiological studies(6,25). But Most studies have not been confirmatory. Overall informations show inconsistent relationships between serum cholesterol levels and cancer risks. Some investigators suggest that low serum cholesterol level might be a metabolic or nutritional consequence of cancer rather than a risk factor(26). Interestingly in this study, SO feeding lowered serum and liver cholesterol. Particularly, carcinogen treatment significantly increased both serum and liver cholesterol levels in CO fed groups while not significantly increased in SO fed groups. Vitamin E supplementation did not affect serum and hepatic cholesterol levels. From these results, we tried to correlate the data between cholesterol levels and GST-P^+ foci formation. As shown in Fig. 4, there was a positive correlation between serum cholesterol and hepatic GST-P^+ foci area. Similar to our findings, Hsing et al.(8) reported that liver cancer mortality is positively correlated with serum cholesterol level in Chinese.

These results imply that SO may have a reducing effect on rat hepatic carcinogenesis by modulating cholesterol metabolism. Kawata et al.(10) reported that the activity of active form of HMG-CoA reductase was increased in human hepatocellula carcinoma(HCC) compared with control subjects. They also found that the rate of cholesterol biosynthesis in HCC was significantly higher than that of normal liver tissue. Thus it seems that the increase of cholesterol synthesis in human HCC partly results from an increase in the active form of the reductase. Increased synthesis and a higher content of cholesterol in HCC than in the normal liver tissue have been described in experimental animals and humans(11). Several lines of evidence(10) have indicated that HMG-CoA reductase activity and the rate of sterol biosynthesis are positively correlated with DNA synthesis and proliferation of mammalian cell.

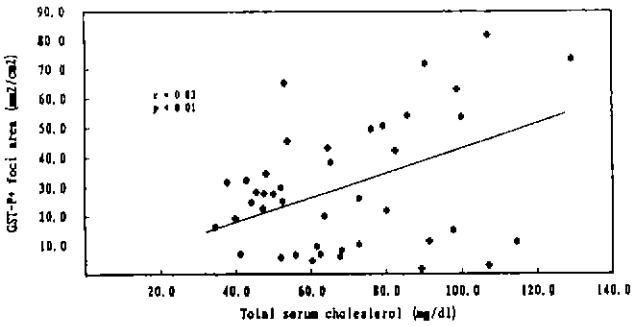

Fig. 4. Correlation between total serum cholesterol and GST-P^+ foci in carcinogen treated rats.
r=correlation coefficient

In conclusion, sardine oil rich in n-3 fatty acid showed an inhibitory effect on the formation of GST-P^+ foci of the liver while vitamin E supplementation did not show any protective effect in this study. These results suggest that the inhibitory effect of n-3 fatty acid on carcinogenesis might be in part due to the alteration of cholesterol biosynthesis. But further research is needed to elucidate the exact mechanisms for such inhibitory effect of n-3 fatty acid on hepatocarcinogenesis.

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