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Effects of Dietary Perilla Oil and Corn Oil on Hepatic Mixed-Function Oxidase System and Antioxidant Enzyme Activities in 2-acetylaminofluorene-treated Rat

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Abstract: This study was conducted to compare the effects of n-6 linoleic acid and n-3 linolenic acid on lipid peroxidation and the activities of enzymes defending against oxidation, which are involved in the tumor promotion, and histological changes of hepatocarcinogen treated rat liver. In this study, weanling male Sprague-Dawley rats were fed one of three diets, containing 15% (w/w) of beef fat (BF), corn oil (CO) or perilla oil (PO), for 11 weeks. During the 3rd week, experimental groups were injected with 2-AAF (50 mg/kg of BW) intraperitoneally 3 times. Findings show that the corn oil diet group has greater liver MDA content than the beef fat and perilla oil diet groups. Also, it is observed that the perilla oil diet group has lower MDA content than beef fat and corn oil diet groups, even though perilla oil is more desaturated than beef fat and corn oil. In terms of activity, mixed-function oxidase activity is not significantly affected by the different dietary fats and 2-AAF treatment. GSH-peroxidase, GSH-reductase and GSH-S-transferase activities are significantly higher in the CO+AAF group than those of the other groups. GST and GSH-Px are activated by 2-AAF treatment in the corn oil diet group only. The hepatocytes of the BF+AAF group were the most severely degenerated, the second was the CO+AAF group and the least was the PO+AAF group. It was also found that dietary corn oil increased lipid peroxidation and activated defense enzymes against oxidation in liver, but dietary perilla oil did not, or supressed defense enzymes. Therefore it is concluded that dietary n-3 linolenic acid in perilla oil inhibits lipid peroxidation and carcinoenesis in rat liver following 2-AAF treatment.

Key words: 2-acetylaminofluorene, glutathione peroxidase, glutathione-S-transferase, malondialdehyde, mixed-function oxidase system.

It has been suggested that a large proportion of human cancers results from exposure to chemical procarcinogens. The type and amount of fat ingested determines the occurrence and development of chemically induced tumors of different organs including the panreas, mammary glands, colon, skin and liver (Roebuck *et al.*, 1981; Reddy and Maruyama, 1986). High fat diets elevate the toxic effects of nuclear-damaging agents and carcinogens (Bird and Bruce, 1986) and high intake of unsaturated fatty acids increases tumor incidence and growth more than high intake of saturated fatty acids. Dietary soybean oil, corn oil and safflower oil, rich in n-6 polyunsaturated fatty acids (PUFA), promotes tumor growth compared with beef tallow (Pearce and Dayton, 1971; Floyd *et al.*, 1976; Summerfield and Tappel, 1984), but fish and perilla oil, rich in n-3 PUFA, have adverse effects (Otamiri and Sjodahl, 1989; Davison and Wills, 1974).

A number of hypotheses has been proposed rearding the action linking dietary fat and cancer. One hypothesis proposes the involvement of free radicals in cell damage and carcinogen activation. Consequences of free radical damage have been implicated in both the initiation and promotion stages of carcinogenesis (Sun, 1990). The interaction of oxygen or reactive oxygen species such as H_2O_2, O_2^- or OH^- with unsaturated fatty acids can promote lipid peroxidation. Lipid peroxidative processes have been linked to diabetes, coronary heart disease, cancer and the aging process (Seis, 1991). Furthermore, reactive aldehydes formed after lipid peroxidation may function as cocarcinogenic agents by being highly cytotoxic and inhibiting protective enzyme functions such as DNA repair (Krokan *et al.*, 1985). One of the most important enzyme systems for metabolically activating carcinogens is a group of

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enzymes known as the mixed-funtion oxidase system located in the endoplasmic reticulum of hepatocyte. This membrane system requires cytochrome P-450, NADPH cytochrome P-450 reductase and phosphatidylcholine for its maximal catalytic activity. Dietary factors such as fat may affect the rate of carcinogen activation or detoxification.

Cell membranes are most susceptible to oxidation because of high PUFA in phospholipids. Therefore, cells have developed several lines of defense against the oxidative attack. The most important defense systems elaborated by the cell are as follows: (1) scavenger enzymes of free radicals or peroxides such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px), glutathione-S-transferase (GST); (2) reducing substances such as reduced glutathione (GSH), cystein, vitamin C, tocopherols, catotene, etc. (Ames, 1983). Corrocher *et al.* (1986) observed that the antioxidant system of the hepatocellular carcinoma cell was severely impaired. Glutathione-S-transferase (GST) detoxifies the external toxic substances by conjugating with GSH, and also has a part in GSH-Px activity. It is activated by carcinogens and lipid peroxides (Sato, 1988).

Dietary fats are known to influence lipid composition of body membranes, depending on their constitutive unsaturated and saturated fatty acid contents. Dietary n-3 PUFAs are efficiently incorporated into tissue membrane phospholipids displacing n-6 PUFAs, such as arachidonic and linoleic acids (Carman and Beare-Rogers, 1988; Nalbone and Termine, 1988). Evidence abounds regarding the effect of dietary n-3 PUFA of fish and seafoods in ameliorating a number of diseases, such as atherosclerosis, heart diease, thrombosis, tumor growth and immune-related pathophysiologies (Herold and Kinsella, 1986; Kinsella *et al.*, 1990).

Traditionally, the apparent consumption of perilla oil is rather high in Korea. Perilla oil has more than 60% of linolenic acid (18:3, n-3) and a high P/S ratio of more than 9 (Hwang and Go,1982). Linolenic acid is metabolically converted to EPA (20:5, n-3) and DHA (22:6, n-3) and has been hypothesized to have some of the beneficial effects like EPA and DHA, rich in fish oil. The objective of the present study was to determine the effects of different types of dietary fatty acids on hepatic lipid peroxidation, and levels of the protective enzymes against the oxidative stress resulting from hepatocarcinogen (2-acetylaminofluorene) treatment.

Materials and Methods

Animals and diet

Weanling male Sprague-Dawley rats were randomly housed in plexiglass cages. They were divided into three groups (twenty per group) and fed on each of the three experimental diets containing 15% (w/w) of beef fat, corn oil or perilla oil (Table 1 and 2) for the period of eleven weeks. Because corn oil contains approximately 31.5 mg α-Tocopherol eqivalent (α-TE)/100 g and perilla oil contains only 0.75 mg α-TE/100 g, α-tocopherol was added to perilla oil (100 mg/100 g oil) based on a peroxidizability index (Witting and Horwitt, 1964) to control for peroxidation and tocopherol effect.

During the 3rd week, half of the rats in each group were injected with 2-acetylaminofluorene (AAF, 50

Table 1. Composition of experimental diet (g/100 g diet)

Ingredient/Diet	Beef fat (BF)	Corn oil (CO)	Perilla oil (PO)
Corn starch	54.7	54.7	54.7
Vitamin-free casein	20.0	20.0	20.0
α-Cellulose	5.0	5.0	5.0
Vitamin mixture[a]	1.0	1.0	1.0
Salt mixture[b]	4.0	4.0	4.0
DL-Methionine	0.3	0.3	0.3
Beef tallow	15.0	–	–
Corn oil	–	15.0	–
Perilla oil	–	–	15.0
α-Tocopherol	–	–	0.015

[a]Nutritional Biochemicals. ICN Science Group. Clevland, Ohio. Vitamin mixture is composed of: vit. A acetate (500,000 IU/g) 1.8 g, Vit. D conc. (850,000 IU/g) 0.125 g, Alpha-Tocopherol (250 IU/g) 22.0 g, Ascorboc acid 45.0 g, Inositol 5.0 g, Choline chroride 75.0 g, Menadione 2.25 g, p-Aminobenzoic acid 5.0 g, Niacin 4.25g, Riboflavin 1.0 g, Pyridoxine hydrochloride 1.0 g, Calcuim pantothenic acid 3.0 g, Biotin 0.02 g, folic acid 0.09 g, Vit. B12 0.00135 g and Dextrose to 1 kg.
[b]Composotion od Salt mixture (g/kg mixture): $CaHPO_4$ 500 g, [b]NaCl 74 g, K_2SO_4 52 g, Potassium citratr monohydrate 22 g, MgO 24 g, Manganese carbonate 1.6 g, Cupric carbonate (53~55% Cu) 0.3 g, KIO_3 0.01 g, Chromium potassium sulfate 0.55 g, $Na_2SeO_3 \cdot 5H_2O$ 0.01 g, Sucrose finely powered 118.0 g.

Table 2. Fatty acid composition of diets (g/100 g diet)

	BF	CO	PO
Saturated fatty acid	7.13	2.73	1.22
Monounsaturated fatty acid	6.03	4.26	2.04
Polyunsaturated fatty acid	0.55	7.98	11.75
18:2 (n-6)	0.46	7.85	2.18
18:3 (n-3)	0.09	0.13	9.57
n-3/n-6	0.20	0.02	4.39
P/S (polyunsaturated/saturated)	0.08	2.92	9.63

BF : Beef fat diet, CO : Corn oil diet, PO : Perilla oil diet.

mg/kg of body weight) in polyethyleneglycol 300 intraperitoneally 3 times at 2-day intervals, and the control group was injected with polyethyleneglycol 300 without 2-AAF. After 8 weeks from the day of first injection, the rats were sacrificed.

Sampling and tissue preparation

After 11 weeks of the feeding experiment, rats were fasted overnight and decapitated. As soon as the liver was removed, two different parts of the left and right lobe were fixed with 10% formalin solution, and stored under refrigeration for hematoxylin-eosin staining.

The rest of liver was homogenized in ice-cold tris-HCl buffer (pH 7.4), centrifuged at 12,000×*g*, 4℃ for 20 min, and the supernatant was centrifuged again at 105,000 ×*g*, 4℃ for 1 h. The upper cytosolic fraction was transferred into several vials and the lower microsomal pellet was resuspended in tris-HCl buffer. The cytosolic and microsomal fractions were frozen with liquid nitrogen and stored at −20℃.

Lipid peroxidation

The thiobarbituric acid (TBA) test, which measures the production of malondialdehyde (MDA), was used to assay lipid peroxide content from the microsomal fraction (Wills, 1987).

Activities of hepatic microsomal mixed-function oxidase system

Cychrome P-450 content was determined by the method of Mastsubara *et al.* (1976) and NADPH-cytochrome P-450 reductase activity was determined by the method of Masters *et al.* (1967).

Activities of GSH-peroxidase, GSH-reductase and GSH-S-transferase

GSH-Px activity was determined by the modified coupled method of Tappel (1978) from the hepatic cytosol fraction using cumene hydroperoxide as the substrate. GSH-reductase activity was determined by the method of Carlberg and Mannervick (1985) from the cytosol fraction. GST activity was determined by the method of Habig (1974) from the cytosol fraction, using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate.

Tissue protein concentration was determined by the method of Lowry *et al.* (1951).

Histological change of hepatocyte

From the fixed liver in formalin solution, hematoxylin and eosin-stained slides were prepared. The degenerative changes were observed with a microscope.

Statistical analysis

Statistical analysis of the data was performed using ANOVA and Duncan's multiple range test at $p<0.05$. The statistical program used was SAS procedures.

Results and Discussion

Growth rate and liver weight

No significant differences in the final body weight and liver weight were found among the six groups. However, the final body weight and growth rate tended to be decreased by 2-AAF treatment in all of the three diet groups

Malondialdehyde content in liver microsome

The CO diet group had the greatest content of MDA in the liver microsomal fraction and the PO diet group had the smallest MDA content. The PO+AAF group had significantly lower MDA content than the other groups (Table 3). The hepatic lipid peroxidation was decreased by the intake of perilla oil, though perilla oil has a much higher P/S ratio than corn oil or beef fat (Table 2).

Intake of corn oil, soybean oil, safflower oil or fish oil was observed to increase lipid peroxides in liver, plasma and urine in several studies (Yim, 1988; Choi, 1990; Iritani *et al.*, 1980; L'abbe *et al.*, 1991; Kim and Choi, 1992). But Hornstra *et al.* (1981) reported that MDA contents in tissue and platelet were decreased by dietary fish oil. Kim and Choi (1992) reported that when the rats were fed the different fat diets for seven weeks, P/S ratios in hepatic microsomal fatty acid composition were found to be 0.83 in the BF group, 1.11 in the CO group and 0.83 in the PO groups, and oleic acid (18:1) and linoleic acid (18:2)

Table 3. Malondialdehyde production in liver microsome

Diet group	Subgroup	MDA (nmol/mg protein)
Beef Fat	BF	$0.48\pm0.15^{ABC a}$
	BF+AAF	0.60 ± 0.23^{AB}
Corn oil	CO	0.58 ± 0.17^{AB}
	CO+AAF	0.65 ± 0.16^{A}
Perilla oil	PO	0.42 ± 0.10^{BC}
	PO+AAF	0.35 ± 0.14^{C}

Values are mean± SD.

[a]Means with the same letter are not significantly different at $p<0.05$ by Duncan test.

BF : Beef fat diet, BF+AAF : Beef fat diet+AAF treatment, CO : Corn oil diet, CO+AAF : Corn oil diet+AAF treatment, PO : Perilla oil diet, PO+AAF : Perilla oil diet+AAF treatment.

Table 4. Cytochrome P-450 content and NADPH-cytochrome P-450 reductase activity in hepatic microsome

Group	Cytochrome P-450 content (nmol/mg protein)	NADPH-cytochrome reductase activity (nmol DCIP/mg protein/min)
BF	0.50 ± 0.23^{Aa}	11.26 ± 1.67^{B}
BF+AAF	0.38 ± 0.24^{A}	13.42 ± 3.69^{AB}
CO	0.50 ± 0.36^{A}	13.79 ± 3.90^{AB}
CO+AAF	0.69 ± 0.28^{A}	13.89 ± 4.34^{AB}
PO	0.54 ± 0.24^{A}	12.92 ± 2.79^{AB}
PO+AAF	0.43 ± 0.23^{A}	16.88 ± 2.90^{A}

Values are mean± SD.
[a]Means with the same letter are not significantly different at $p<0.05$ by Duncan test.

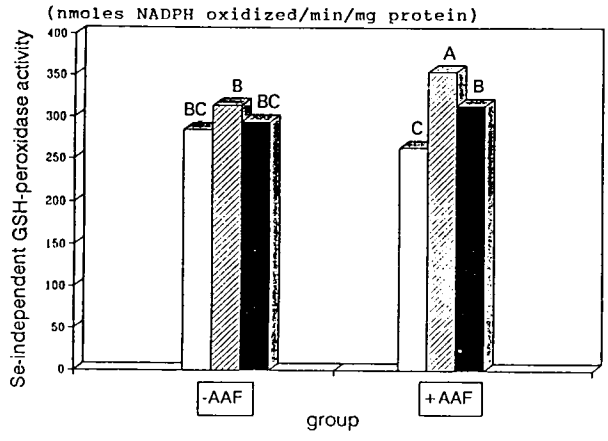

Fig. 1. Se-independent glutathione peroxidase activity in the rat liver. □ Beef fat. ▨ Corn oil, ■ Perilla oil.

decreased, arachidonic acid (20:4) increased and the MDA content was decreased by 2-AAF treatment in all of the three diet groups.

The cholesterol/phospholipid ratio decreased (Kim, 1988), and the PUFA content in phosphoatidylcholine (Davison and Wills, 1974) and the MDA content increased (Yim, 1988; Kensler and Trush, 1984) in hepatic microsome following some carcinogen treatments. However, in this study, no significant effect on MDA content is found in hepatic microsome following 2-AAF treatment.

Hepatic microsomal mixed-function oxidase system activity

Table 4 shows that hepatic microsomal cytochrome P-450 content and NADPH-cytochrome P-450 reductase activity were not significantly affected by the different dietary fats and 2-AAF treatment.

The cytochrome P-450-dependent liver microsomal mixed function oxidase system plays a pivotal role in the metabolism of a wide variety of drugs and foreign compounds. The mixed function oxidases are membrane bound and phosphatidylcholine is necessary for proper function of the system (Anderson *et al.*, 1982). Variations in dietary lipids with different fatty acids alter the fatty acid composition of phoapholipids of the microsomal membranes and the activity of the mixed function oxidases. Kim *et al.* (1990) suggested with regard to the regulation of cytochrome P-450 by dietary lipids that polyunsaturated fatty acids affect some signal involved in the de novo synthesis of proteins. Several studies indicate that elevation of the proportion of polyunsaturated fatty acids in dietary lipids increased the activity of mixed function oxidases (Wade and Norred, 1976; Clinton *et al.*, 1984). However, intake of polyunsaturated fatty acids has been observed as having no effect on some of the mixed function oxidases (Meydani *et al.*, 1985). Yim (1988) and Kim *et al.* (1990) observed that 2-AAF treatment enhanced the cytochrome P-450 content in beef fat or soybean oil diet rats. Saito *et al.* (1990) reported that NADPH-cytochrome P-450 reductase activities in the lard and sardine oil groups were not influenced by large doses of the dietary lipids (25%), although the activity of the 5% sardine oil group was significantly higher than that of the 5% lard group.

Activities of GSH-peroxidase, GSH-reductase and GSH-S-transferase

GSH-Px not only catalyzes the conversion of lipid hydroperoxides released from membranes by phospholipase A_2 to corresponding fatty acids, but also reduces hydrogen peroxide. GSH-Px in rat liver exhibits much less activity toward H_2O_2 as compared to organic hydroperoxides (Meister and Anderson, 1983).

Fig. 1 shows that GSH-Px activity in the liver cytosol function was not significantly effected by the different dietary fats without 2-AAF treatment. However, significant difference among the BF+AAF, CO+AAF and PO+AAF groups were observed. CO+AAF group had the highest activity of Se-independent GSH-Px, which is hypothesized to be stimulated to protect against the increased oxidative stress of linoleic acid (n-6) in corn oil. The GSH-Px activity of the PO+AAF group was significantly higher than that of the BF+AAF group, but lower than that of the CO+AAF group. Linolenic acid (n-3) in perilla oil might not have induced oxidative stress as much as linoleic acid (n-6). These results confirm the findings that hepatic GSH-Px activity and lipid peroxides of soybean oil diet rats were significantly higher than those of beef fat diet rats (Yim, 1988), and GSH-Px activity was depressed in rats fed high fat diets compared with rats fed a low fat diet (Connye

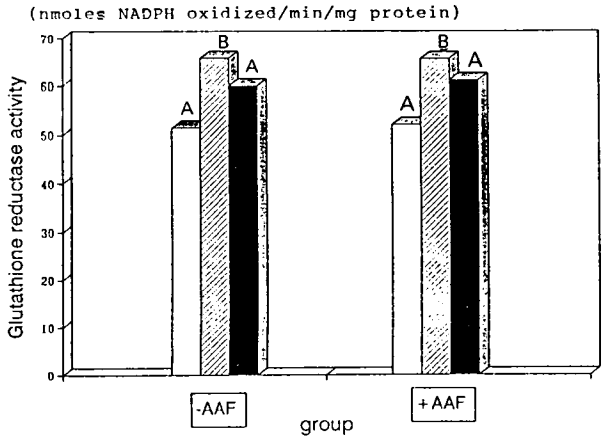

Fig. 2. Glutathione reductase activity in the rat liver.
□ Beef fat. ▨ Corn oil, ■ Perilla oil.

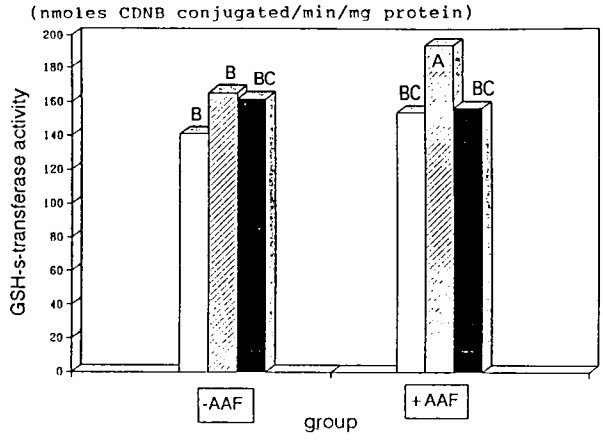

Fig. 3. Glutathions-S-transferase activity in the rat liver.
□ Beef fat. ▨ Corn oil, ■ Perilla oil.

and Barbara, 1991). L'Abbe *et al.* (1991) reported that dietary menhaden oil elevated the urinary TBARS but reduced the hepatic GSH-Px activity in rats.

Fig. 1 also shows that Se-independent GSH-Px activity was elevated by 2-AAF treatment in the CO diet group, but not in the BF and PO diet groups. Kim and Choi (1992) reported that hepatic GSH-Px activity was elevated by 2-AAF treatment in both beef tallow diet and soybean oil diet rats.

GSH-reductase is a NADPH-dependent enzyme and keeps GSH level by trnaferring electrons to oxidized GSH (GSSG) (Seis, 1991). The activity of GSH-Px is positively correlated with the content of GSH in normal liver (Corrocher *et al.*, 1986). GST plays an important role in detoxification by catabolizing the conjugation of many hydrophilic and electrophiic substances with reduced glutathione (GSH) and also has Se-independent GSH-Px activity (Sato, 1988; Schramm *et al.*, 1981). Several studies report that GST activity is elevated by high intake of corn oil (Kwei *et al.*, 1991), soybean oil and/or carcinogen treatment (Sato, 1988, Astrom and DePierre, 1981; Kim *et al.*, 1990; Kim and Choi, 1992)

In this study, GSH reductase activity (Fig. 2) and GST avtivity (Fig. 3) were significantly elevated by dietary corn oil with and without 2-AAF treatment, but not by dietary perilla oil. GSH-Px, GSH reductase and GST might be activated by the increased lipid peroxides, free radicals or electrophilic 2-AAF metabolites in the corn oil diet group.

Treatment with linoleic acid in cultured porcine endotherial cells increased GSH-reductase activity and GSH-Px activity by 37% and 136%, respectively, compared with controls. But linolenic acid is not toxic to endothelial cells in contrast to linoleic acid (Toborek and Hennig, 1994).

Microscopy of hepatocyte

Table 5 shows the prevalences on 10 steps to hepatoma and the mean score by the Han's method (1979). No tumor was found but hydropic degeneration and some fatty changes in the three basal diet groups without 2-AAF treatment was observed and there was no significant difference among them (Table 5). However, the hepatocytes of the rats fed corn oil or beef fat diet were more severely degenerated by 2-AAF treatment and there was a significantly different effect due to different dietary fats with 2-AAF treatment. The hepatocytes of the BF+AAF group were the most severely degenerated, the second was the CO+AAF group and the least was the PO+AAF group. More severe changes, such as balooning degeneration, portal fibrosis and ovalocyte proliferation were observed following 2-AAF treatment in the BF and CO diet groups, but not in the PO diet group. When the rats were exposed to the same level of carcinogen, the rats fed the PO diet were the most resistent to the degenerative changes in hepatocytes. Kim (1994) reported that rats fed a corn oil diet marked significantly higher score in hepatocyte degeneration and larger placental glutathione-S-transferase positive foci area than rats fed perilla oil or sardine oil.

Conclusion

High intake of corn oil elevated the MDA content in hepatic microsome, especially in 2-AAF treated rats, and also activated antioxidant enzymes such as GSH-Px, GSH reductase and GSH-S-transferase. However, high intake of perilla oil did not. Histological degeneration of hepatocyte due to 2-AAF treatment was found to be the mildest in the perilla oil diet group.

Therefore, it may be concluded that dietary n-3 lino-

Table 5. Prevalence of histological degeneration in the rat liver

Histological change	BF(10)[a]	BF+AAF(10)	CO(10)	CO+AAF(10)	PO(9)	PO+AAF(9)
Hydropic degeneration	2	0	5	3	8	7
Fatty change	1	1	3	3	0	1
Prebalooning degeneration	0	3	0	4	1	5
Balooning degeneration	3	7	2	2	0	0
Bile duct proliferation	0	5	0	0	0	0
Portal fibrosis	2	6	1	4	0	0
Ovalocyte proliferation	0	3	0	1	0	0
Pseudo lobule	0	0	0	0	0	0
Hyperplastic nodule	0	0	0	0	0	0
Hepatoma	0	0	0	0	0	0
Mean score[b]	2.6 ± 1.2^{Cc}	12.8 ± 1.2^{A}	2.6 ± 0.9^{C}	6.4 ± 1.3^{B}	1.2 ± 0.4^{C}	2.9 ± 0.4^{C}

[a]Number of rat.
[b]values are mean± SE., Scoring: hydropic degeneration (+ 1.0, ++ 1.3, +++ 1.6), Fatty change (+ 2.0, ++ 2.3, +++ 2.6), Prebalooning degeneration (+ 3.0,++ 3.3,+++ 3.6), Balooning degeneration (+ 4.0, ++ 4.3,+++ 4.6), Bile duct proliferation (+ 5.0, ++ 5.3, +++ 5.6), Portal fibrosis (+ 6.0, ++ 6.3, +++ 6.6), Ovalocyte proliferation (+ 7.0, ++ 7.3, +++ 7.6), Pseudo lobule (+ 8.0, ++ 8.3, +++ 8.6), Hyperplastic nodule (+ 9.0, ++ 9.3, +++ 9.6), Hepatoma (+ 10.0, ++ 10.3, +++ 10.6).
[c]Means with the same letter are not significantly different at $p<0.05$ by Duncan test.

lenic acid in perilla oil inhibits the lipid peroxidation-induced tumor promotion from 2-AAF exposure, but dietary n-6 linoleic acid in corn oil stimulates it.

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