Effect of Dietary Fats (Perilla Oil, Lard, Rapeseed Oil) on Peroxidizability of Mouse Brain Lipids

Junji Terao,* Akihiko Nagao, Hiramitsu Suzuki, and Megumi Yamazaki†

National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki 305, Japan

The effect of dietary fats (perilla oil, lard, and rapeseed oil) on the susceptibility of brain lipids to lipid peroxidation in male ICR mice was investigated. The brain weight and the brain lipid content were not changed by the intake of diet containing 6% (by weight) different dietary fats after a 15-month feeding period. The number of bisallylic hydrogens of brain lipids was significantly increased by the intake of α-linolenic acid rich perilla oil. The concentration of α-tocopherol in the brain lipids from the mice fed perilla oil containing diet was also higher than those from the mice fed rapeseed oil containing diet and the mice fed standard chow diet. Intake of perilla oil has little effect on the lipid peroxidation status of the brain homogenates, as measured by thiobarbituric acid assay and the rate of oxygen absorption induced by a free-radical initiator. It is therefore suggested that dietary intake of perilla oil does not elevate the susceptibility of brain lipids toward free radical driven lipid peroxidation in spite of its lability to oxidative deterioration, when dietary α-tocopherol is sufficiently supplied.

INTRODUCTION

Oxidative deterioration of edible oil lowers the nutritional value and causes off-flavors. The toxicity of oxidized oil has also been well recognized in recent years. In addition, the influence of dietary fats and oils on the lipid peroxidation status in vivo has attracted much attention because dietary fats and oils are more or less responsible for the composition of membrane lipids in all tissues and organs (Hammer and Wills, 1978; Iritani et al., 1980). Fish oils contain highly unsaturated and potentially unstable $n-3$ fatty acids such as eicosapentaenoic acid and docosahexaenoic acid. Consumption of fish oil is believed to be effective in lowering the incidence of coronary heart disease (Herold and Kinsella, 1986; Lands, 1986). However, considerable studies have suggested that the high intake of this oxidizable oil elevates the susceptibility to lipid peroxidation in vivo (Draper et al., 1984; Mounie et al., 1986; Nalbone et al., 1988, 1989; Piche et al., 1988; Hu et al., 1989; Leibovitz et al., 1990).

Perilla oil extracted from perilla seeds (*Perilla frutescens* var. crispa) contains high level of α-linolenic acid, a highly oxidizable $n-3$ fatty acid. However, requirement of α-linolenic acid has been linked to the development of brain function (Benolken et al., 1973; Lamptey and Walker, 1976; Yamamoto et al., 1987). As compared to other organs, the brain seems to be highly susceptible to oxidative stress because of high consumption of oxygen and high contents of unstable polyunsaturated fatty acids (PUFAs). Lipid peroxidation induced free-radical damage is suspected to have a deleterious effect leading to pathological conditions in brain tissue (Floyd, 1988). Thus, the question is raised as to whether the intake of highly oxidizable oils such as perilla oil affects the susceptibility of brain tissue to lipid peroxidation. Nevertheless, little is known about the effect of dietary oils on the lipid peroxidation status of brain tissue.

The present study was conducted to investigate this question by evaluating the peroxidizability of brain lipids after long-term intake of edible oils with different oxidative stability. Male mice were fed diets containing perilla oil, lard, and rapeseed oil for 15 months. The fatty acid compositions and tocopherol contents in the extracted brain lipids were analyzed and their values compared.

* Address correspondence to this author.
† Present address: Iwate Prefectural Brewing and Food Research Institute, Tonan, Shiwa, Iwate 020, Japan.

EXPERIMENTAL PROCEDURES

Animals and Diets. Male Crj:CD-1(ICR) mice (5 weeks old) were obtained from Charles River Japan Inc. (Atsugi, Kanagawa, Japan). Forty male mice were divided into four groups of 10 and housed in polycarbonated cages (44 × 28 × 18 cm). The animals were maintained in an environmentally controlled room with 12-h periods of light and dark (Suzuki et al., 1989). They were placed on perilla oil diet, lard diet, rapeseed oil diet, and standard chow diet for 15 months. The first three diets were prepared by us and contained 6% lipid sources (gifts from Tsukishima Food Co., Tokyo). The remaining components were as follows: corn starch, 41.5%; casein, 25%; α-starch, 10.0%; cellulose powder, 8.0%; granulated sugar, 5.0%; salt mixture (AIN-76), 3.5%; vitamin mixture (AIN-76 plus choline bitartrate), 1.0%. Salt and vitamin mixtures were purchased from Oriental Yeast Co. Ltd., Tokyo). Each diet contained α-tocopherol acetate (5 mg/100 g of diet). To prevent lipid peroxidation during storage, each experimental diet was inserted into a pouch with an oxygen absorber (Ageless S-200, Mitsubishi Gas Chemical Co. Inc., Tokyo; activated iron oxide packed with an air-permeable packaging material) and stored at 5 °C (Suzuki et al., 1985). Standard chow diet was obtained from Oriental Yeast. This diet contained lipid source (5.6%) and α-tocopherol (9.1 mg/100 g of diet). The diet and water were provided ad libitum. At the end of the feeding trial, the mice were fasted for 24 h and killed by decapitation. The whole brain was removed immediately, washed with physiological saline, and stored in a glass vial at −60 °C (Ebara low temperature freezer ES-300, Ebara Co., Tokyo) until used, but no longer than 1 month after harvesting. Six samples were randomly selected from each group and used for the experiments.

Lipid Extraction. Total lipids were extracted from mouse brain according to the method of Bligh and Dyer (1959) with slight modification. The whole brain was minced and homogenized by using a Physicotron homogenizer (NS-310E, Nition, Chiba, Japan) in ice-cold 0.1 M Tris-HCl buffer (pH 7.4) containing 0.135 M KCl. To this homogenate (1.5 mL, 10% w/v) was added 5.0 mL of chloroform/methanol (1:2 v/v) with vigorous mixing. The mixture was then centrifuged at 3500 rpm for 5 min. The supernatant was removed and saved. To the precipitate was added 6.3 mL of methanol/chloroform/water (2:1:0.8 v/v/

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v) with vigorous mixing. After the centrifugation (3500 rpm, 5 min), the supernatants were combined, diluted with 5.3 mL of chloroform and 5.3 mL of methanol, and mixed vigorously. The mixture was centrifuged at 3500 rpm for 10 min. The lower chloroform phase was withdrawn and an additional 5.3 mL of chloroform was added to the upper methanol–water phase. This was centrifuged for 10 min. The lower layers were combined and evaporated in vacuo until the lipid weight was constant. Total lipids obtained were redissolved in 1.5 mL of hexane/2-propanol (3:2 v/v) and stored in the freezer (−20 °C).

Determination of Fatty Acid Compositions. The fatty acid compositions of total lipids of mouse brain or edible oils were determined by gas chromatographic analysis after transmethylation. The solution of total lipids (0.2 mL) was evaporated with dry nitrogen gas, and then 5% HCl in methanol (0.4 mL) was added to the residue in a screw-capped tube. After the mixture was heated at 100 °C for 1 h, water (0.5 mL) and hexane (0.5 mL) were added, and the mixture was centrifuged (3500 rpm, 5 min). The hexane layer was concentrated to 0.1 mL under dry nitrogen, and 1 μL of solution was injected to the column. A Shimadzu GC-7A gas chromatograph (Shimadzu Co., Kyoto) was used with a fused silica capillary column DB-225 (J&W Scientific Inc., Folsom, CA). The operational column temperature was kept at 200 °C. Sample solution was injected by using a solvent cut injector (SCI, Gaskuro Kogyo Inc., Tokyo). A Shimadzu Chromatopac C-R1A was used for the calculation of peak areas in the chromatogram.

Determination of Tocopherols. To the stored solution of total lipids (1.0 mL) was added a known amount of pentamethylchroman as an internal standard followed by evaporation in vacuo. When completely dry, 100 μL of ethanol was added with careful mixing, and a 20-μL sample was injected into the HPLC. HPLC analysis was performed with a Shimadzu LC-4A device and a Shimadzu spectrofluorometer RF-500 (excitation, 298 nm; emission, 325 nm). A YMC-packed ODS column (6 × 150 mm, 5-μm particle size; Yamamura Kagaku Co., Kyoto) was used for the analytical separation and a guard column (5 × 20 mm) packed with the same packing material was used to prevent contamination of the analytical column. The column was eluted by acetonitrile/2-propanol (4:1 v/v) at a flow rate of 2.0 mL/min.

Thiobarbituric Acid (TBA) Assay and Oxygen Absorption of Brain Homogenate. A part of the mouse brain homogenate (1.0 mL, 10% w/v) was centrifuged at 3000 rpm for 10 min at 4 °C. A 0.5-mL sample of supernatant was removed and diluted with 0.5 mL of 0.1 M Tris-HCl containing 0.135 M KCl. The sample solution was incubated at 37 °C in a rotary water bath (Cutler, 1985). After incubation was completed, the TBA assay was carried out according to the method of Uchiyama and Mihara (1978). On the other hand, the residual supernatant was used for the measurement of oxygen absorption by a free radical generating system. The supernatant was 20 times diluted by 0.1 M Tris-HCl (pH 7.4) containing 0.135 M KCl. The diluted solution was put into a cell (4 mL volume), and its temperature was maintained at 37 °C. An aliquot of 2,2′-azobis(2-amidinopropane) hydrochloride (AAPH) was injected to the cell (final concentration, 20 mM) to initiate radical chain oxidation of the brain homogenate (Niki, 1990). Oxygen absorption was measured by using an oxygen electrode (Rank Brothers, Booltusham, Cambridge, U.K.) at 0.8 V.

Measurement of Oxidative Stability of Edible Oils. A 1-g sample of each oil was measured accurately and spread out in a glass vial (55-mm diameter). This was stored at 60 °C in the dark with circulating air. The weight gain was recorded at an appropriate interval (Olcott and Einset, 1958).

Statistical Method. The results of these experiments are presented as means ± SD for six animals. Student's *t*-test at the 0.05 significant level was used to examine the statistical significance of the mean responses among each group.

RESULTS

Table I shows the fatty acid compositions of edible oils used for the experimental diets. In perilla oils, α-linolenic acid (18:3, $n-3$) occupied nearly 50% of total fatty acids. Lard and rapeseed oil were rich in monounsaturated fatty acids, oleic acid (18:1), and erucic acid (22:1), respectively.

Table I. Fatty Acid Compositions of the Dietary Oils[a]

fatty acid	mol %: perilla oil	lard	rapeseed oil	standard chow
14:0	ND	1.9	ND	T
16:0	6.9	26.9	3.9	15.5
16:1	ND	3.1	T	1.5
18:0	2.8	14.0	1.4	2.5
18:1	19.0	41.8	17.5	23.3
18:2 ($n-6$)	15.4	9.3	15.2	48.8
18:3 ($n-6$)	8.0	T	T	ND
18:3 ($n-3$)	48.2	T	9.1	3.7
20:0	ND	T	T	ND
20:1	ND	T	8.2	T
20:5 ($n-3$)	ND	ND	ND	1.5
22:1	ND	ND	41.4	ND
22:6 ($n-3$)	ND	ND	ND	1.2
($n-3/n-6$)	2.06	0.05	0.57	0.12
$C{=}CCH_2C{=}C$	2.40	0.21	0.69	1.36

[a] T, trace amount (less than 1%). ND, not detected.

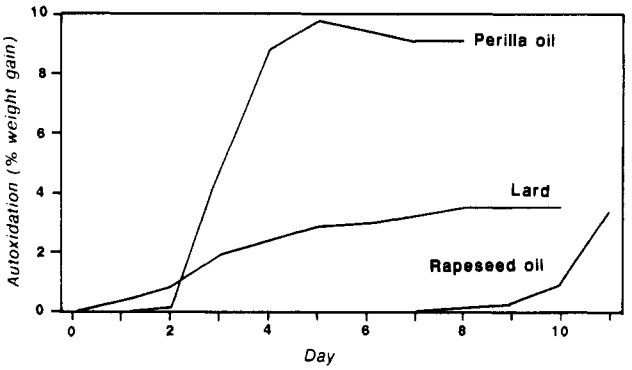

Figure 1. Autoxidation of the dietary oils at 60 °C.

The ratios of $n-3$ fatty acids to $n-6$ fatty acids ($n-3/n-6$) increased in the following order: lard < rapeseed oil < perilla oil. The ratios obtained from perilla oil were found to be much higher than that from the fatty acid composition of standard chow diet.

Linoleic acid (18:2, $n-6$) containing two double bonds and α-linolenic acid (18:3, $n-3$) containing three double bonds each possess two bisallylic hydrogens at the C-11 position and the C-11 and C-14 positions, respectively. Similarly, PUFA containing n double bonds possesses $2(n-1)$ bisallylic hydrogens in its structure. The total number of bisallylic hydrogens in the dietary oils ($C{=}CCH_2C{=}C$) could be calculated by the equation

$$(C{=}CCH_2C{=}C) = [\Sigma\ (\%\ \text{of PUFA containing}\ n\ \text{double bonds} \times 2(n-1))] \times 10^{-2}$$

Perilla oil exhibited the highest value among the three oils. In addition, the value for perilla oil was found to be much higher than that obtained from the fatty acid compositions of standard chow diet. The content of tocopherols was determined as follows: perilla oil 0.02 (α), 0.33 (β + γ) 0.01 (δ) mg/g of oil; rapeseed oil 0.19 (α), 0.34 (β + γ), 0.01 (δ) mg/g of oil. No significant level of tocopherols was detected in lard. Perilla oil and lard were much less stable than rapeseed oil when they were allowed to autoxidize at 60 °C in bulk phase (Figure 1). No induction period was observed in the oxidation of lard, because of the lack of tocopherols. However, its oxidation rate was slower than that of perilla oil.

One animal on the perilla oil diet, one animal on the rapeseed oil diet, two animals on the lard diet, and one

Table II. Fatty Acid Compositions and Tocopherol Content in Brain Lipids[a]

fatty acid	mol % perilla oil diet	lard diet	rapeseed oil diet	standard chow diet
16:0	22.2 ± 0.1[a]	25.3 ± 1.0[b]	26.2 ± 1.8[b]	23.6 ± 0.7[c]
16:1	1.4 ± 0.2	T	T	T
18:0	14.0 ± 1.7[a]	19.9 ± 3.3[b]	16.7 ± 5.2[ab]	18.8 ± 3.1[b]
18:1	22.7 ± 0.7[ac]	21.1 ± 1.3[b]	20.0 ± 1.3[ab]	22.6 ± 1.1[c]
18:2 ($n-6$)	1.1 ± 0.5	1.2 ± 1.0	ND	1.1 ± 0.4
20:1	2.1 ± 1.4	1.6 ± 0.6	1.8 ± 1.0	2.1 ± 0.6
20:3 ($n-6$)	T	ND	ND	ND
20:3 ($n-3$)	T	ND	ND	ND
20:4 ($n-6$)	9.2 ± 0.8	10.7 ± 1.8	10.5 ± 2.4	10.5 ± 1.7
20:5 ($n-3$)	T	ND	ND	ND
22:0	T	ND	ND	ND
22:4 ($n-6$)	1.2 ± 0.2[a]	2.4 ± 1.2[bc]	1.1 ± 0.9[ab]	2.3 ± 0.7[c]
22:5 ($n-3$)	T	ND	ND	ND
22:6 ($n-3$)	21.5 ± 1.6[a]	14.6 ± 2.7[b]	16.6 ± 3.5[bc]	18.2 ± 2.8[c]
unidentified	ND	3.6 ± 1.8	4.7 ± 1.5	ND
($n-3/n-6$)	1.96 ± 0.19[a]	1.00 ± 0.15[b]	1.34 ± 0.25[c]	1.30 ± 0.08[c]
$C=CCH_2C=C$	2.98 ± 0.19[a]	2.25 ± 0.32[b]	2.41 ± 0.40[b]	2.61 ± 0.35[b]
α-tocopherol content, nmol/g of lipids	109 ± 45[a]	61 ± 30[ac]	25 ± 20[b]	35 ± 9[bc]
α-Toc/$C=CCH_2CH=CH$, $\times 10^{-6}$	11.7 ± 4.0[a]	7.5 ± 3.9[ab]	3.0 ± 2.5[b]	4.6 ± 1.6[b]

[a] Average value ± SD for six animals. Values carrying different superscripts in one line are significantly different. T, trace amount (less than 1%). ND, not detected.

animal on the standard chow diet died during the feeding trial. After a 15-month feeding period, no significant difference was observed in the brain weights of surviving mice: 0.50 ± 0.03, 0.50 ± 0.03, 0.52 ± 0.03, and 0.50 ± 0.02 g for perilla oil diet, lard diet, rapeseed oil diet, and standard chow diet groups, respectively. The brain lipid contents were also not significantly different: 82 ± 6, 78 ± 7, 74 ± 6, and 72 ± 5 mg/g for perilla oil diet, lard diet, rapeseed oil diet, and standard chow diet groups, respectively. Table II shows the fatty acid compositions and tocopherol contents in brain lipids extracted from the mice of each diet group. Major PUFAs in brain lipids were arachidonic acid [20:4($n-6$)] and docosahexaenoic acid [22:6($n-3$)]. In addition, docosatetraenoic acid [22:4($n-6$)] was present as a minor component in all groups. The order of ($n-3/n-6$) was lard diet group < rapeseed oil diet group < perilla oil diet group, similar to the dietary oils.

The number of bisallylic hydrogens in brain lipids from the mice fed perilla oil diet was found to be greater than those from the mice fed the other two diets. This value was also significantly different from that of the mice fed the standard chow diet. Among tocopherols, only α-tocopherol was detected in the brain lipids of all groups. Concentrations of α-tocopherol in brain lipids of mice fed the dietary oil varied widely, compared with that of mice fed the standard chow diet. However, the perilla oil diet group exhibited a significantly higher level of α-tocopherol than the standard chow diet group.

To evaluate the oxidative stability of brain lipids, the ratio of α-tocopherol content to the number of bisallylic hydrogens (α-Toc/$C=CCH_2C=C$) was calculated by the equation

$$(\alpha\text{-Toc}/C{=}CCH_2C{=}C) = \{\alpha\text{-tocopherol content (mol/g)} \times \text{average molecular weight of brain lipids}\}/\text{total number of bisallylic hydrogens}$$

The average molecular weights of brain lipids were calculated from their fatty acid compositions. The ratios were not greatly different among the diet groups as shown in Table II. However, the value obtained from the perilla oil diet group was significantly higher than that from the rapeseed oil diet group. This trend was the reverse of the oxidative stability of the edible oils shown in Figure 1.

Table III. Formation of Thiobarbituric Acid Reactants from Brain Homogenate before and after Incubation at 37 °C for 16 h[a]

incubation	μmol of malondialdehyde/g of tissue perilla oil diet	lard diet	rapeseed oil diet	standard chow diet
before	0.11 ± 0.03[a]	0.08 ± 0.02[a]	0.24 ± 0.06[b]	0.09 ± 0.01[a]
after	0.76 ± 0.09[a]	0.71 ± 0.08[a]	0.99 ± 0.09[b]	0.92 ± 0.04[b]

[a] Average value ± SD for six animals. Values carrying different superscripts in one line are significantly different.

Table IV. Oxygen Absorption ($\mu M\ s^{-1}$) of Brain Homogenate Induced by a Water-Soluble Radical Initiator[a]

perilla oil diet	lard diet	rapeseed diet	standard chow diet
0.08 ± 0.02[a]	0.07 ± 0.03[a]	0.10 ± 0.03[ac]	0.11 ± 0.02[bc]

[a] Average value ± SD from six animals. Values carrying different superscripts in one line are significantly different.

Table III shows the result of thiobarbituric acid (TBA) assay of the brain homogenate obtained from each diet group. Whether with or without incubation, the levels of TBA reacting substances in the brain homogenates from the mice fed perilla oil diet and lard diet were significantly lower than that from the mice fed oxidation-resistant rapeseed oil. When the radical initiator was introduced into the diluted homogenate, dissolved oxygen was linearly decreased without a clear induction period. Thus, the susceptibility of brain homogenate toward free radicals was measured by the rate of oxygen absorption as shown in Table IV. There were no significant differences among the three diet groups, although the perilla oil diet group and the lard diet group exhibited lower values than the rapeseed oil diet group.

DISCUSSION

In general, nonenzymatic lipid peroxidation leading to tissue damage proceeds via free-radical chain reaction. In this process, bisallylic hydrogen in esterified and nonesterified PUFA is exclusively abstracted by the lipid peroxyl radical (LOO•), resulting in the formation of lipid

hydroperoxides (Howard and Ingold, 1963):

$$C{=}CCH_2C{=}C + LOO^{\bullet} \rightarrow C{=}CC^{\bullet}HC{=}C + LOOH \quad (1)$$

Cosgrove et al. (1987) have demonstrated by kinetic experiment that the oxidizability of PUFA is linearly dependent on the number of bisallylic positions. It is therefore reasonable to use the number of bisallylic hydrogens as an index of the oxidizability of tissue lipids to free radical driven lipid peroxidation. Although either the saturation/unsaturation ratio (Buckingham, 1985) or the index calculated from the number of double bonds in each fatty acid constituent (Witting and Horwitt, 1964; Arakawa and Sagai, 1986) has been frequently used as the index of peroxidizability of tissue lipids, we believe that the number of bisallylic hydrogens is a more reliable index on the kinetic basis. On the other hand, α-tocopherol (TocH) is known to inhibit radical chain reaction by scavenging chain-propagating lipid peroxyl radicals (Burton and Ingold, 1981; Niki et al., 1984):

$$LOO^{\bullet} + TocH \rightarrow LOOH + Toc^{\bullet} \quad (2)$$

It has been suggested that this lipophilic vitamin is primarily responsible for the oxidative stability of brain regions against lipid peroxidation (Meydani et al., 1985a, 1988). In fact, the higher rate of the peroxidation of brain microsomes is linked to its lower ratio of α-tocopherol concentration to peroxidizable PUFAs (Kornbrust and Mavis, 1980). Therefore, we propose the ratio of α-tocopherol to bisallylic hydrogens as a parameter for evaluating the oxidative stability of brain lipids to free radical driven lipid peroxidation.

Our results on the fatty acid compositions of mouse brain lipids confirmed that fatty acid composition of brain lipids in rodent animals can be modified by the long-term intake of certain dietary oils (Foot et al., 1982; Bourre et al., 1990). This modification also leads to changes of the $(n-3/n-6)$ ratio and the number of bisallylic hydrogens in the fatty acid of the mice fed these dietary oils. In our experiment, intake of perilla oil significantly elevated the number of bisallylic hydrogens in the brain lipids, as compared to that with the standard chow diet. Interestingly, α-tocopherol content in brain lipids was significantly increased by the intake of perilla oil. This results in a higher ratio of α-tocopherol to bisallylic hydrogens in brain lipids from the mice fed the perilla oil diet than the ratio from the mice fed the standard chow diet (Table II). We have also found that free-radical damage by lipid peroxidation, as measured by the production of TBA reacting substance and the rate of oxygen absorption, was not elevated in the brain tissue from the mice fed perilla oil as compared with those fed lard, rapeseed oil, and standard chow diets. This implies that the intake of perilla oil does not increase the risk of lipid peroxidation induced free-radical damage.

The concentration of α-tocopherol in brain tissue depends on dietary α-tocopherol (Meydani et al., 1988). In our experiments, the dietary oils might contribute to the concentration of α-tocopherol in the diet which contained α-tocopherol acetate (5 mg/100 g of diet). However, dietary α-tocopherol from perilla oil (0.02 mg/g of oil) was much less than that from rapeseed oil (0.19 mg/g of oil). Thus, our results strongly suggest that certain dietary oils can affect the concentration of α-tocopherol in brain lipids when α-tocopherol is supplied from the diet. Determinants for the concentration of α-tocopherol in tissue arise from the process of absorption and/or that of postabsorptive utilization of dietary α-tocopherol. Mouri et al. (1984) reported that intake of eicosapentaenoic acid and docosahexaenoic acid rich fish oil decrease α-tocopherol content in the plasma and liver of rats. Meydani et al. (1987) confirmed that α-tocopherol levels in mouse plasma, liver, kidney, and lung were lowered by the intake of fish oil and suggested the loss of α-tocopherol appeared to occur during the absorption process. It is likely that PUFA decreases the absorption of tocopherols at the intestine (Andeson et al., 1971; Gallo-Forres, 1980; Elmadha and Schwalbe, 1989). However, this may not be the case for α-linolenic acid, because we have suggested that intake of α-linolenic acid rich perilla oil did not decrease α-tocopherol levels in brain lipids. Dietary α-linolenic acid is known to exert a protective action in vitamin E deficiency syndrome, chick nutritional encephalomalacia (Dam et al., 1958; Budowski and Crawford, 1986). Intake of α-linolenic acid rich perilla oil may affect the postabsorptive utilization of dietary α-tocopherol such as the regulation of eicosanoid synthesis (Meydani et al., 1985b). Further studies are necessary to fully understand the influence of dietary oil on the accumulation of α-tocopherol in brain lipids.

In conclusion, dietary intake of α-linolenic acid rich perilla oil does not elevate the susceptibility of brain lipids toward free-radical peroxidation in spite of its high oxidizability, when α-tocopherol is sufficiently supplied from the diet. This may be because the α-tocopherol level in brain lipids increases with the intake of perilla oil. The influence of dietary oil on the peroxidizability of brain lipids seems to be of more complicated nature than that expected from the oxidizability of dietary oils.

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