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Development of Alcoholic Fatty Liver and Fibrosis in Rhesus Monkeys Fed a Low n-3 Fatty Acid Diet

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Background: The amount and type of dietary fat seem to be important factors that modulate the development of alcohol-induced liver steatosis and fibrosis. Various alcohol-feeding studies in animals have been used to model some of the symptoms that occur in liver disease in humans.

Methods: Rhesus monkeys (*Macaca mulatta*) were maintained on a diet that had a very low concentration of α-linolenic acid and were given free access to an artificially sweetened 7% ethanol solution. Control and ethanol-consuming animals were maintained on a diet in which the linoleate content was adequate (1.4% of energy); however, α-linoleate represented only 0.08% of energy. Liver specimens were obtained, and the fatty acid composition of the liver phospholipids, cholesterol esters, and triglycerides of the two groups were compared at 5 years and histopathology of tissue samples were compared at 3 and 5 years.

Results: The mean consumption of ethanol for this group over a 5-year period was 2.4 $g \cdot kg^{-1} \cdot day^{-1}$. As a consequence of the ethanol-dietary treatment, there were significantly lower concentrations of several polyunsaturated fatty acids in the liver phospholipids of the alcohol-treated group, including arachidonic acid and most of the n-3 fatty acids and particularly docosahexaenoic acid, when compared with dietary controls. Liver specimens from animals in the ethanol group at 5 years showed a marked degree of steatosis, both focal and diffuse cellular necrosis, and an increase in the development of fibrosis compared with specimens obtained at 3 years and with those from dietary controls, in which there was no evidence of fibrotic lesions.

Conclusion: These findings suggest that the advancement of ethanol-induced liver disease in rhesus monkeys may be modulated by the amount and type of dietary essential fatty acids and that a marginal intake of n-3 fatty acids may be a permissive factor in the development of liver disease in primates.

Key Words: Liver Fibrosis, Rhesus Monkeys, α-Linolenic Acid, Arachidonic Acid, Docosahexaenoic Acid.

THE DEVELOPMENT OF alcoholic liver disease (ALD) in humans has a complex etiology, which involves a broad range of sequential and simultaneous biochemical and cellular events that are influenced by both genetically determined and environmental factors (Britton and Bacon, 1994; Hu et al., 1997; Lands, 1995; Lieber, 2000; Shimada et al., 1994). Because inadequate nutrition is often a major compounding factor that intensifies the development of ALD (Bode and Bode, 1992; French, 1993; Halsted et al., 2002a; Lands et al., 1999; Mezey, 1991; Thomson and Pratt, 1992), a considerable body of research has focused on the effects that specific dietary constituents have on the development and/or treatment of liver injury in humans and in animal model systems of ALD (Halsted et al., 2002b; Lieber, 2002; Lieber et al., 1994; Matsuoka et al., 1990; Nanji et al., 1994, 2003; Pawlosky et al., 1997; Tsukamoto et al., 1986).

The amount and the type of the dietary fat seem to be important factors that often modulate the development of alcohol-induced liver pathologies, and various alcohol-feeding studies in animals have been used to model some of the symptoms that occur in ALD in humans (Halsted et al., 2002b; Lieber et al., 1994; Nanji et al., 1989, 1994, 2001; Pawlosky et al., 1997; Tsukamoto et al., 1986). For example, using an intragastric tube-feeding method of alcohol delivery, rodents that were maintained on diets in which a high percentage of energy was derived from fat (35 en%) that either contained high concentrations of linoleic acid (18:2n6) (Nanji and French, 1989) or was composed entirely of menhaden fish oil [containing both eicosapentaenoic (20:5n3) and docosahexaenoic acids (22:6n3)] developed fatty liver, inflammation, and fibrosis (Nanji et al., 1994, 2001). Conversely, diets that contained high amounts of saturated fats seemed to protect alcohol-fed animals from liver damage (Nanji et al., 2001). It was suggested that unusually high concentrations of polyunsaturated fatty acids (PUFA) may accentuate alcohol-induced lipid peroxidation, activating

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NF-κB, up-regulating tumor necrosis factor-α, and triggering production and deposition of collagen (Nanji and French, 1989; Nanji et al., 2001). These findings are in contrast with several studies in humans that show that fish oil supplementation generally suppresses proinflammatory cytokine activation when compared with n-6 fatty acid supplement (Caughey et al., 1996; Endres et al., 1989; Kelley et al., 1999; Meydani et al., 1991). Thus, it remains unclear whether under physiological conditions n-3 fatty acids (or their metabolites) potentiate alcohol-induced injury or act to suppress inflammation and thereby protect the liver from injury (Baybutt et al., 2002; Hayashi et al., 1999).

An important determinant of the effects of prolonged alcohol exposure on liver function is the manner in which PUFA and/or their oxygenated derivatives (e.g., eicosanoids) are altered. Prolonged alcohol consumption has been shown to deplete liver phospholipids of PUFA in several species (Anggard et al., 1983; Cunningham et al., 1983; Nakamura et al., 1992; Pawlosky and Salem, 1995, 1999; Salem and Olsson, 1997; Salem and Ward, 1993). Ethanol consumption depleted 18:2n6, 20:4n6, and 22:6n3 in the livers of felines and in rhesus monkeys when animals were maintained on low essential fatty acid (EFA) diets (Pawlosky and Salem, 1995, 1999). Furthermore, research in baboons has suggested that an alcohol-induced loss of liver polyunsaturated phosphatidylcholine was associated with the development of septal fibrosis and cirrhosis (Lieber et al., 1994).

Previously, we reported that when rhesus monkeys were maintained on a diet that was generally nutritionally adequate but had a low n-3 fatty acid content (18:3n3 was 0.08 en%) and were given free access to an ethanol solution for 18 months, they developed moderate steatosis and pericellular fibrosis (Pawlosky et al., 1997). The dietary controls had essentially normal livers. This report describes the effects that alcohol feeding has in combination with a diet with low n-3 fatty acid content on the liver lipid composition and pathology in rhesus monkeys after 3 and 5 years.

MATERIALS AND METHODS

Animal Procedures

Experimental details of the study have been reported previously (Pawlosky et al., 1997) and are only briefly described here. Animal procedures were carried out according to the National Institutes of Health animal welfare guidelines, and the protocol was approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee. Ten adult male rhesus monkeys (*Macaca mulatta*; 8–16 kg) that were free of known pathogens were housed separately, acclimated to a semipurified diet (Bio-Serv Inc., Frenchtown, NJ; Table 1), and given access to food twice a day. After 1 month of dietary equilibration, six animals were given 24-hr access to an artificially flavored 7% ethanol solution that was sweetened with aspartame for 5 years. Peak blood alcohol concentrations (BACs) were determined using an alcohol dehydrogenase kit (Sigma Chemical Co., St. Louis, MO) after a period of alcohol consumption. Control animals were maintained on the same diet and provided with the same amount of food as the alcohol group (540 kcal/day).

Table 1. Nutrient Content and Fatty Acyl Composition of the Primate Diet

Nutrient	g/kg
Casein vitamin-free	176
Corn starch	425
Sucrose	201
Fat[a]	
Olive oil	20
Hydrogenated coconut oil	40
Alphacel nonnutritive bulk	30
Mineral mixture[b]	
Vitamin mixture[c]	
Vitamin E	40 IU/kg
Vitamin C	250 mg/kg

[a] Fatty acid, weight percentage 12:0, 6.2; 14:0, 1.8; 16:0, 18.3; 16:1n7, 0.5; 18:0, 11.8; 18:1n9, 54.4; 18:1n7, 1.0; t18:1n9, 0.1; 18:2n6, 8.4; 18:3n6, 0.4; 18:3n3, 0.5; 20-carbon PUFA were not detected or were <0.01%.

[b] All values expressed in g/kg of diet or as otherwise noted: calcium, 5; chlorine, 4.6; chromium, 0.5 mg; copper, 3.8 mg; fluorine, 0.5 mg; iodine, 3.6 mg; iron, 0.249; manganese, 0.02; phosphorus, 3.76; potassium, 7.32; selenium, 0.06 mg; sodium, 3.37; sulfur, 0.67; zinc, 0.02; magnesium, 1.50.

[c] All values expressed in g/kg of diet or as otherwise noted: biotin 0.2 mg; pantothenate, 0.025; choline, 2.5; folic acid, 6.35 mg; inositol, 0.52; menadione, 1.5 mg; niacin, 42 mg; pyridoxine, 4.0 mg; riboflavin, 8.0 mg; thiamine, 4.0 mg; vitamin A, 18,000 IU; vitamin D3, 1300 IU; vitamin B-12, 0.2 mg; para-aminobenzoic acid, 0.25.

Diet

Before the study, animals had been maintained on a commercial primate diet (Purina Lab Diet, St. Louis, MO). The fatty acyl composition of this diet is as follows: 18:2n6 (44.6 wt%), 18:3n6 (0.22 wt%), 20:4n6 (0.11 wt%), 18:3n3 (2.6 wt%), 20:5n3 (0.49 wt%), and 22:6n3 (0.68 wt%). The composition of the experimental diet is given in Table 1. The fat content (14 en%) of the diet consisted of a 2:1 ratio of olive oil/hydrogenated coconut oil and contained no 20- or 22-carbon PUFA. There were 8.9 g of 18:2n6 and 0.5 g of 18:3n3 per 100 g of fat. The levels of vitamins C (250 mg/kg) and E (40 IU/kg) were set at the National Research Council recommendation level for this species (Bourne, 1975).

Liver Specimens

Liver specimens at 3 years were obtained by ultrasound-guided needle-punch biopsy as previously reported, and at 5 years, animals were killed, whole livers were removed, and sections of liver were obtained. Specimens were frozen or fixed in 10% formalin. Liver specimens were sectioned and stained with hematoxylin and eosin, Masson's Trichrome, or silver stain for reticulin fibers. Tissue processing was carried out by American Histo Labs (Gaithersburg, MD). Liver samples that were intended for biochemical analysis were frozen and stored at −80°C until processed.

Histopathology and Specimen Grading

Specimens were graded with respect to fat accumulation and collagen deposition by an experienced veterinarian pathologist who was unaware of the treatment that the animals received. A mild (grade 1) fat score indicated that 10 to 15% of the hepatocytes contained vacuoles that rendered a positive stain for lipid (oil red O); moderate (grade 2) was assigned when vacuolization increased to 15 to 30% of the hepatocytes with the appearance of both micro- and macrovesicular droplets; and marked (grade 3) was assigned when the fat was distributed in >40% of the hepatocytes. Fibrosis was classified with respect to tissue localization and complexity. Pericellular fibrosis was evidenced by the appearance of reticulin fibers (silver stained for collagen material) that formed a lattice-shaped network enclosing clusters of hepatocytes (Rabin, 1989). Central and periportal venular fibrosis was characterized by the appearance of densely Trichrome-staining material that encircled a central vein or the circumference of a portal triad. Septal and bridging fibrosis was evidenced by the appearance of densely staining collagen material that converged with a collagen branch at a juncture forming a bridge or septa.

Fatty Acyl Analyses of Liver Specimens

Frozen pieces of liver (100 mg) were thawed, weighed, and then analyzed for their fatty acyl compositions. Tissue samples were homogenized in water and methanol, and the lipids were extracted into chloroform (Bligh and Dyer, 1959). An internal standard, 23:0 methyl ester (50 μg/sample) was added to liver samples to quantify the absolute amounts of fatty acids. Phospholipids, cholesterol esters, and triglycerides were isolated using a solid-phase cartridge procedure as previously described (Kim and Salem, 1990). The lipid extracts were converted to the methyl esters using boron trifluoride in methanol (14% w/v), according to the method of Morrison and Smith (1961). Fatty acid methyl esters were then analyzed by gas chromatography with flame ionization detection as has been described previously (Salem et al., 1996).

Data Reduction and Statistical Analyses

Concentrations of the liver fatty acids are reported in μg/g wet weight because there were changes in the total lipid concentration of the tissues in alcohol-exposed animals. The fatty acyl profiles of the phospholipids, cholesterol esters, and triglycerides are expressed in mole percentage (mol %) of the total fatty acids. The Student's t test was used to determine the difference between the mean values of the weight percentages or absolute concentrations of fatty acids. A $p \leq 0.05$ was considered significant.

RESULTS

The mean daily alcohol intake over the 5-year period was 2.4 g $\cdot$ kg^{-1} $\cdot$ day^{-1} (range: 1.8–4.6 g $\cdot$ kg^{-1} $\cdot$ day^{-1}) or 24% of the total daily calories (range: 16–42%). Control animals were maintained on the same diet, and food intake was monitored daily. Animals in both groups were generally healthy and consumed all of their daily food rations. Although alcohol-fed animals received a greater number of calories, this did not seem to have a clear positive effect on body weight (Pawlosky and Salem, 1999).

As a result of the low EFA content of the diet, the concentrations of some of the PUFA in the livers from animals in both the alcohol and dietary control groups were very low compared with adult animals that were maintained continuously on the commercial diet (Purina Lab Diet). The proportion of PUFA in the liver phospholipids of animals that subsisted on the commercial diet was 18:2n6 (20.3 wt%), 20:4n6 (9.31 wt%), 20:5n3 (0.30 wt%), 22:5n3 (1.2 wt%), and 22:6n3 (7.8 wt%) compared with values from our dietary control animals: 18:2n6 (10.9 wt%), 20:4n6 (8.65 wt%), 20:5n3 (0.31 wt%), 22:5n3 (0.41 wt%), and 22:6n3 (1.8 wt%). The proportion of 20:4n6 and 20:5n3 was similar in both groups of animals; however, there was a 4-fold lower percentage of 22:6n3 in the livers of animals in the dietary control group compared with animals on the commercial diet. Moreover, the ratio of n-3/n-6 fatty acids in the phospholipids of animals that were maintained on the commercial diet was 0.33 compared with 0.12 in animals that were maintained on the experimental diet.

Alcohol consumption further compounded the effect of the low EFA diet because the PUFA concentrations in the livers of the ethanol-fed animals were significantly lower than those from the dietary control group (Table 2). The concentrations (μg/g) of 18:2n6 (1532 $\pm$ 90 vs. 1039 $\pm$ 94; $p < 0.001$), 20:4n6 (965 $\pm$ 44 vs. 542 $\pm$ 25; $p < 0.0001$), 22:5n6 (120 $\pm$ 19 vs. 62 $\pm$ 8; $p < 0.05$), 18:3n3 (27 $\pm$ 2 vs. 12 $\pm$ 2; $p < 0.001$), 20:5n3 (29 $\pm$ 3 vs. 18 $\pm$ 2; $p < 0.05$), 22:5n3 (49 $\pm$ 4 vs. 25 $\pm$ 1; $p < 0.0001$), and 22:6n3 (181 $\pm$ 18 vs. 60 $\pm$ 7; $p < 0.0001$) all were markedly lower in the livers of alcohol-fed animals compared with dietary controls. There did not seem to be any clear effect of alcohol on the concentrations of nonessential fatty acids.

Table 2. Fatty Acyl Composition of Liver Total Lipid Extracts From Dietary Control and Ethanol-Consuming Rhesus Monkeys After 5 Years

Fatty acid	Control[a] (n = 4; μg/g)	Alcohol (n = 5; μg/g)
Nonessential		
16:0	2949 $\pm$ 55	2994 $\pm$ 106
16:1	571 $\pm$ 140	738 $\pm$ 32
18:0	1529 $\pm$ 166	1339 $\pm$ 82
18:1n-9	3378 $\pm$ 146	3793 $\pm$ 32
18:1n-7	628 $\pm$ 94	728 $\pm$ 100
20:3n-9	121 $\pm$ 7	133 $\pm$ 16
24:0	34 $\pm$ 4	35 $\pm$ 4
24:1n-9	73 $\pm$ 3	73 $\pm$ 14
Total nonessential	9283 $\pm$ 169	9833 $\pm$ 204
n-6		
18:2	1532 $\pm$ 90	1039 $\pm$ 94[c]
20:3	267 $\pm$ 13	191 $\pm$ 32
20:4	965 $\pm$ 44	542 $\pm$ 25[d]
22:4	52 $\pm$ 7	38 $\pm$ 4
22:5	120 $\pm$ 19	62 $\pm$ 8[b]
Total n-6	2936 $\pm$ 112	1872 $\pm$ 150[c]
n-3		
18:3	27 $\pm$ 2	12 $\pm$ 2[c]
20:5	29 $\pm$ 3	18 $\pm$ 2[b]
22:5	49 $\pm$ 4	25 $\pm$ 1[d]
22:6	181 $\pm$ 18	60 $\pm$ 7[d]
Total n-3	286 $\pm$ 13	115 $\pm$ 6[d]

[a] Data are expressed as the mean value ($\pm$SD) in μg/g of wet weight of liver from animals 5 years after beginning a low n-3 fatty acid diet or low n-3 fatty acid diet with access to an alcohol solution.

Significantly different from controls (Student's t test) [b] $p < 0.05$, [c] $p < 0.001$, [d] $p < 0.0001$.

In the fatty acyl profiles of the liver phospholipids, there were small increases in the percentages of the nonessential fatty acids 16:1 and 24:0 in ethanol-fed animals compared with the controls (Table 3). Consistent with the values from the total lipid analyses, the percentages of 20:4n6, 18:3n3, 22:5n3, and 22:6n3 were lower in the alcohol-consuming animals. In the cholesterol ester fractions from the liver, there were no differences in the percentages of n-6 fatty acids between these groups; however, the percentage of 20:5n3 was lower in the alcohol-fed animals compared with the controls (Table 4). Because of the low n-3 fatty acid content of the diet, the n-3 fatty acids in the triacylglyceride fraction were below the limits of quantification for this method and are not reported. However, there were lower percentages of the major n-6 PUFA in the triacylglycerides in the ethanol group compared with the controls (Table 5). Thus, it seemed that the alcohol-lowering effect on 22:6n3 was primarily localized to the phospholipids, whereas the loss of 20:5n3 was greater in the cholesterol ester fraction.

On examination of the livers, there seemed to be few distinguishing gross morphological differences in the livers of the ethanol-fed animals compared with controls. However, the livers from several of the animals in the alcohol

Table 3. Fatty Acyl Composition of Liver Phospholipids From Control and Ethanol-Consuming Primates After 5 Years

Fatty acid	Control[a] (n = 4; mol %)	Alcohol (n = 5; mol %)
Nonessential		
16:0	26.05 ± 0.93	25.05 ± 0.53
16:1	2.72 ± 0.28	3.70 ± 0.50
18:0	20.29 ± 0.60	18.38 ± 0.24
18:1n-9	16.86 ± 0.82	19.74 ± 1.82
18:1n-7	2.92 ± 1.2	4.45 ± 0.54
20:3n-9	1.11 ± 0.16	1.45 ± 0.20
24:0	0.38 ± 0.02	0.48 ± 0.03[b]
24:1n-9	0.65 ± 0.05	0.74 ± 0.15
n-6		
18:2	12.07 ± 1.67	11.8 ± 0.37
18:3	0.26 ± 0.03	0.24 ± 0.02
20:3	2.64 ± 0.07	2.40 ± 0.51
20:4	8.82 ± 0.35	7.13 ± 0.45[b]
22:4	0.51 ± 0.01	0.48 ± 0.10
22:5	2.21 ± 0.02	1.82 ± 0.23
Total n-6 fatty acids	26.51 ± 1.61	23.87 ± 0.66
n-3		
18:3	0.14 ± 0.04	0.09 ± 0.04[b]
20:5	0.31 ± 0.03	0.22 ± 0.01[b]
22:5	0.41 ± 0.10	0.27 ± 0.03
22:6	1.77 ± 0.10	0.85 ± 0.10[b]
Total n-3 fatty acids	2.63 ± 0.11	1.43 ± 0.08[b]

[a] Data are expressed as mean weight percentage of total fatty acids (±SD) at 5 years from animals on a low n-3 fatty acid diet or low n-3 fatty acid diet with free access to a sweetened 7% alcohol solution.

[b] Significantly different from controls (Student's t test) $p < 0.05$.

Table 4. Fatty Acyl Composition of Liver Cholesterol Esters From Control and Alcohol-Consuming Primates After 5 Years

Fatty acid	Control[a] (n = 4; mol %)	Alcohol (n = 5; mol %)
Nonessential		
16:0	31.2 ± 4.9	20.4 ± 2.1
16:1	6.54 ± 1.9	8.9 ± 0.7
18:0	7.77 ± 1.1	8.47 ± 1.2
18:1n-9	39.2 ± 2.7	43.9 ± 2.9
18:1n-7	5.5 ± 0.5	6.5 ± 0.9
20:3n-9	0.49 ± 0.01	0.8 ± 0.2
n-6		
18:2	5.72 ± 0.4	7.23 ± 0.8
18:3	0.36 ± 0.04	0.40 ± 0.1
20:3	0.70 ± 0.4	0.6 ± 0.4
20:4	0.70 ± 0.2	0.6 ± 0.1
22:5	0.37 ± 0.2	0.14 ± 0.7
n-3		
18:3	ND	ND
20:5	0.51 ± 0.2	0.31 ± 0.05[b]
22:6	ND	ND

ND, not detected.

[a] Data are expressed as mean weight percentage of total fatty acids (±SD) at 5 years from animals on a low EFA diet or low EFA diet with free access to a sweetened alcohol solution.

[b] Significantly different from controls, $p < 0.05$ (Student's t test).

group were streaked with fat (Fig. 1). None of the livers was cirrhotic.

Figure 2A shows a typical liver section stained with Masson's Trichrome from a control rhesus monkey at 5 years. Liver specimens from control animals on the low n-3 EFA diet generally seemed normal or unremarkable with respect to the development of any discernible pathology. In contrast, typical liver sections from ethanol-fed animals demonstrated some degree of steatosis (hepatocellular vacuolization grade 2 or grade 3), which was diffuse (Fig. 2B). Both macro- and microvesicular fat-containing vacuoles were observed in enlarged hepatocytes, and cells that contained fat vacuoles were often clustered. Only one animal from the ethanol group did not demonstrate noticeable accumulation of fat in any of the liver sections examined, and this animal consumed the lowest amount of ethanol (average alcohol consumption: 1.8 $g \cdot kg^{-1} \cdot day^{-1}$).

Figure 3A shows an example of a liver section that was silver-stained for reticulin fibers from a control animal and an ethanol-fed monkey at 3 years (Fig. 3B). Pericellular fibrosis was observed in the alcohol-fed animals at both 3 and 5 years and was not observed in the controls. The degree to which fibrosis developed varied among individual animals and was more pronounced in animals that consumed the highest amount of alcohol. The animal that

Table 5. Fatty Acyl Composition of Liver Triacylglcerides From Control and Alcohol-Consuming Primates After 5 Years

Fatty acid	Control[a] (n = 4; mol %)	Alcohol (n = 5; mol %)
Nonessential		
16:0	32.82 ± 0.72	33.43 ± 0.79
16:1	8.00 ± 1.63	9.44 ± 0.25
18:0	4.41 ± 0.74	4.65 ± 0.50
18:1n-9	39.31 ± 2.65	41.29 ± 0.84
18:1n-7	5.92 ± 0.60	6.77 ± 0.84
20:3n-9	0.31 ± 0.02	0.23 ± 0.03
n-6		
18:2	7.03 ± 1.05	3.16 ± 0.13[c]
20:3	0.52 ± 0.07	0.23 ± 0.4[c]
20:4	1.28 ± 0.23	0.51 ± 0.06[b]

[a] Data are expressed as mean weight percentage of total fatty acids (±SD) at 5 years from animals on a low n-3 fatty acid diet or low n-3 fatty acid diet with free access to a sweetened alcohol solution. The levels of n-3 fatty acids were not detected (<0.01 wt%).

[b] Significantly different from controls (Student's t test) $p < 0.05$.

[c] $p < 0.01$.

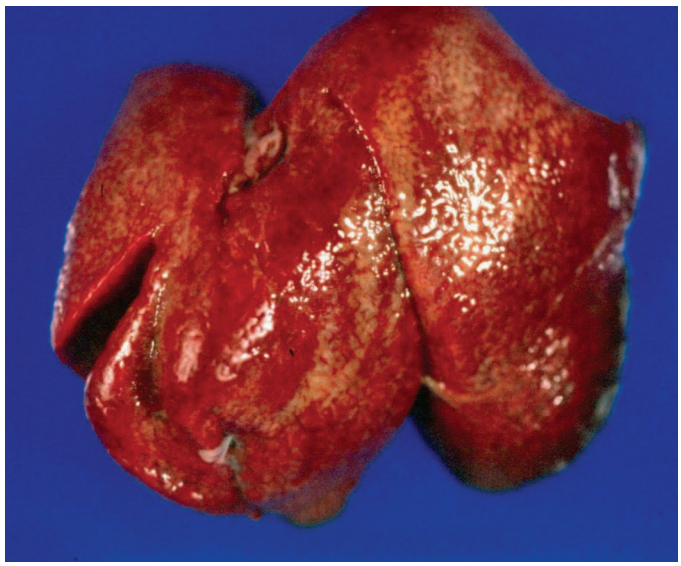

Fig. 1. Liver from an adult ethanol-consuming rhesus monkey after 5 years on a low n-3 fatty acid diet (alcohol consumption: 4.6 $g \cdot kg^{-1} \cdot day^{-1}$). The slide shows a liver that is streaked with fatty veins.

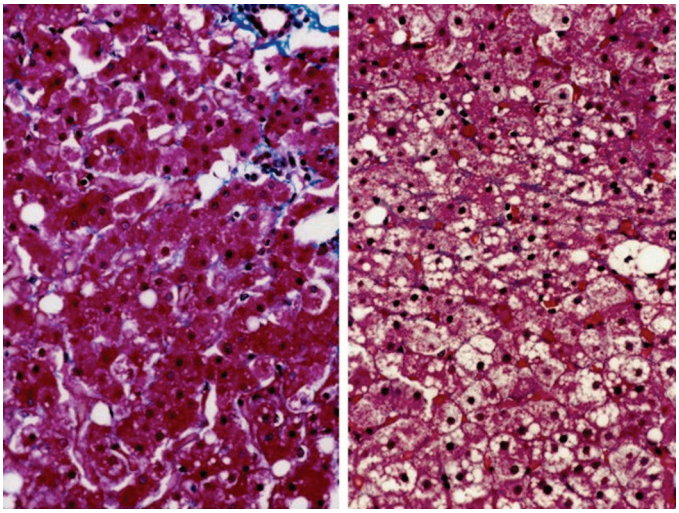

Fig. 2. (A) Liver section from an adult rhesus monkey after 5 years on a low n-3 fatty acid diet and (B) a liver section from an animal after 5 years on a low n-3 fatty acid diet with alcohol consumption. Sections are stained with Masson's Trichrome. (B) Enlarged hepatocytes from alcohol-consuming animal (average alcohol consumption: 4.6 g · kg^{-1} · day^{-1}) that contain both macro- and microvesicular vacuoles that contain fat droplets. Magnification: ×20.

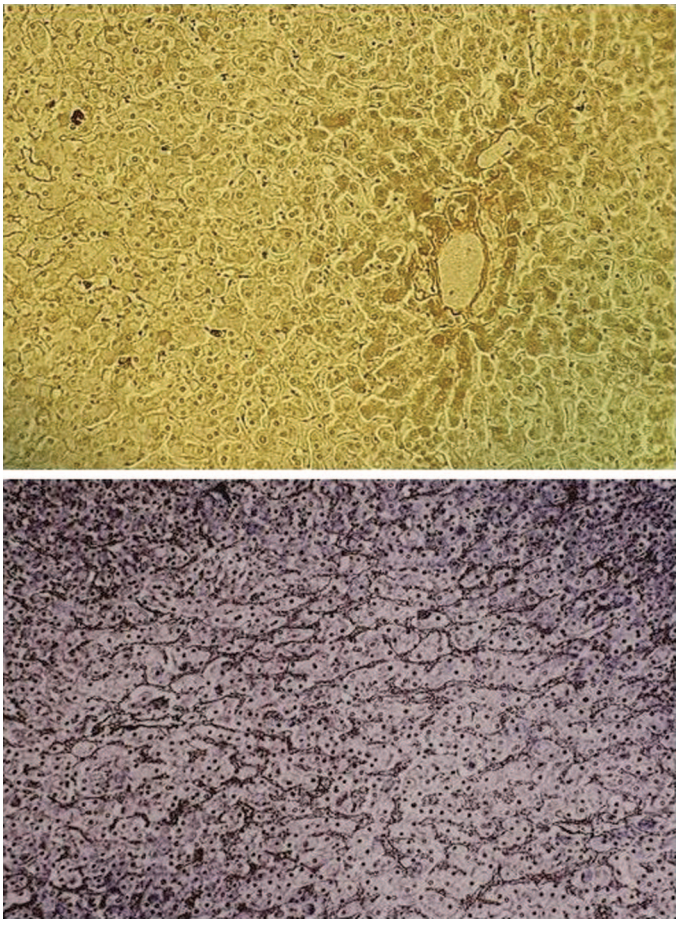

Fig. 3. (A) Liver section from an adult rhesus monkey on a low n-3 fatty acid diet for 3 years and (B) an adult animal on low n-3 fatty acid diet for 3 years with alcohol consumption (alcohol consumption: 2.6 g · kg^{-1} · day^{-1}). The section is silver-stained for reticulin fibers. Reticulin fibers (B) present a cross-linked lattice effect across the section. Magnification: ×40.

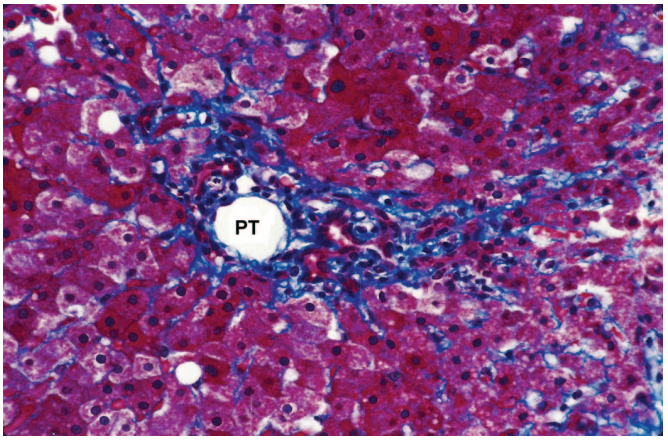

Fig. 4. Liver section is from a rhesus monkey that was maintained on a low n-3 fatty acid diet after 5 years of alcohol consumption (alcohol consumption: 3.3 g · kg^{-1} · day^{-1}). The section is stained with Masson's Trichrome. Collagen surrounds a portal vein (PT) and extends beyond the triad into the tissue. Magnification: ×40.

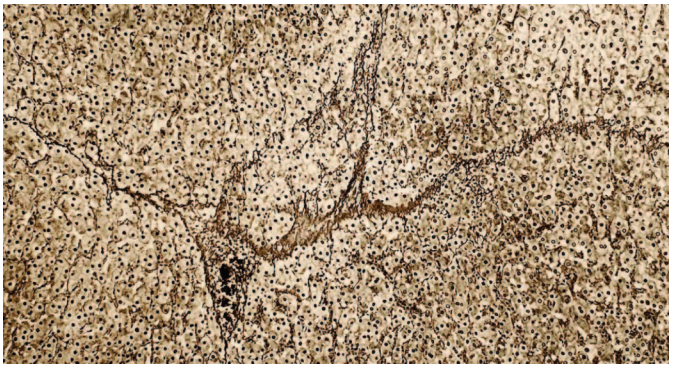

Fig. 5. Liver section from is a rhesus monkey on a low n-3 fatty acid diet after 5 years of alcohol consumption (alcohol consumption: 2.9 g · kg^{-1} · day^{-1}). The section is silver-stained for reticulin fibers. Reticulin fibers extend throughout the tissue. Magnification: ×10.

consumed the least amount of ethanol did not develop fibrosis.

Figure 4 is a liver section from another alcohol-fed animal at 5 years; it was stained with Trichrome, which shows development of periportal venular fibrosis in zone 1 with collagen extending beyond the triad. Central lobular and sinusoidal fibrosis were also observed in this specimen but stained less intensely for collagen.

Figures 5 and 6 depict a liver section obtained from a different alcohol-fed animal at 5 years and demonstrate the occurrence of both focal and diffuse cellular necrosis with fibrosis. Figure 5 (silver-stained section) shows the formation of a reticulin fibrous network extending throughout the tissue. Figure 6 is a higher magnification of the micrograph in Fig. 5 (stained with Trichrome). Hepatocytes seem to be undergoing necrosis, and a dense necrotic lesion can be observed in the central field. To the right of center, a fibrotic septum formed from conjoining collagen branches is visible.

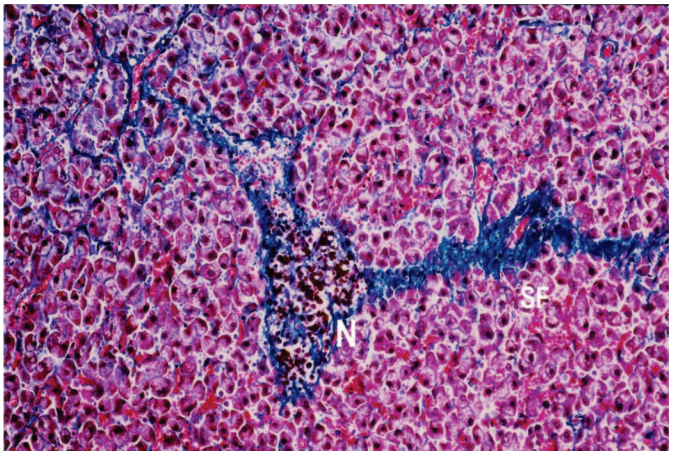

Fig. 6. Magnification of liver section from Fig. 5. The section is stained with Masson's Trichrome. Diffuse and focal (N) cellular necrosis that is surrounded by collagen can be observed. Thicken fibrotic lesions (SF) forma septa upon contact. Magnification: ×40.

DISCUSSION

This model of alcohol-induced liver disease in rhesus monkeys suggests that the development of fibrosis may be attributed, in part, to depletion of tissue PUFA, and this may more specifically involve a depletion of 22:6n3 in liver phospholipids. Consistent with this view, it was hypothesized that a diet with an adequate amount of 18:2n6 but low levels of 18:3n3 might enhance the progression of the liver pathology in animals during a prolonged period of alcohol consumption. Animals that consumed $>1.8\ g \cdot kg^{-1} \cdot day^{-1}$ exhibited fibrosis with varying degrees of complexity after 5 years. Liver pathology consistent with the development of ALD occurred in the livers of most of the alcohol-fed animals at the 3- and 5-year time points (de la M Hall et al., 2001).

Although high concentrations of dietary 18:2n6 or menhaden fish oil were found to cause development of liver pathology in rodents that received ethanol through an intragastric tube (Nanji and French, 1989; Nanji et al. 1994, 2001), we observed that when primates were maintained on a diet that contained relatively low amounts of 18:2n6 and 18:3n3 and regularly consumed alcohol, they progressively developed steatosis and fibrosis. This finding is consistent with alcohol studies in other primates and suggests that depletion of liver EFAs is an important factor that contributes to the development of liver injury in primates. Moreover, diet seems to be an important variable predicting the development of the pathology in primates because alcohol feeding did not induce fibrosis or cirrhosis in baboons that were maintained on a diet that contained a high concentration of lipotropes that included substantial concentrations of EFAs (Ainley et al., 1988).

Although both long-chain n-6 and n-3 fatty acids were lower in the total lipid extracts and in the phospholipids in the alcohol group compared with controls, the concentration of n-3 fatty acids were more sharply affected than n-6 fatty acids (Tables 2 and 3). It is has been shown that long-chain n-3 fatty acids, such as 20:5n3 and 22:6n3 (or their metabolites), generally inhibit proinflammatory processes (reducing tumor necrosis factor-α and proinflammatory cytokines) in tissues, and this further suggests that low levels of dietary n-3 fatty acids may have been an important factor that heightened inflammatory signaling responses (Caughey et al., 1996; Endres et al., 1989; James et al., 2000; Kelley et al., 1999; Meydani et al., 1991).

Recently, the role of the mitochondria in the development of ALD has been reviewed (Adachi and Ishi, 2002; Bailey and Cunningham, 2002). Chronic alcohol consumption has been shown to alter liver mitochondrial function (Cunningham et al., 1983; Fernandez-Checa et al., 1991; Pastorino et al., 1999), and alcohol-induced changes in mitochondrial membrane structure may be involved in events that precede hepatocyte apoptosis. Studies have shown that chronic alcohol consumption can deplete liver mitochondrial membranes of 22:6n3 (Cunningham et al., 1982; Thompson and Reitz, 1978; Villanueva et al., 1994), and animals that are maintained on a diet that has a very low concentration of 18:3n3 would have low concentrations of 22:6n3 in liver mitochondrial membranes.

Only a small percentage (10–20%) of chronic alcoholics develop liver pathology that proceeds to fibrosis or cirrhosis (Bordalo et al., 1974; Bruguera et al., 1977; Rankin et al., 1978). Diet and nutrition are considered important variables that correlate with incidence of occurrence. The EFA content of the rhesus diet was based on olive oil, a vegetable oil commonly used in the Mediterranean region, a region with the world's highest incidence of ALD (Grant et al., 1988). It is interesting that frequent consumption of fish has been associated with a lower incidence of liver cirrhosis in both Japanese men and women (Hirayama, 1990). Although the levels of dietary 18:2n6 was adequate for this species (Nakamura et al., 1992), low amounts of dietary n-3 EFA may, in fact, characterize a marginal diet common to some alcoholics and people who abuse alcohol, which increases the risk of developing an n-3 fatty acid deficiency (Alling et al., 1984; Gruchow et al., 1985; Johnson et al., 1985).

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