Dietary Juniper Berry Oil Minimizes Hepatic Reperfusion Injury in the Rat

SHANNON M. JONES, ZHI ZHONG, NOBUYUKI ENOMOTO, PETER SCHEMMER, AND RONALD G. THURMAN

Juniper berry oil is rich in 5,11,14-eicosatrienoic acid, a polyunsaturated fatty acid similar to one found in fish oil, yet less prone to peroxidation. Dietary fish oil treatment has been shown to effectively reduce reperfusion injury; therefore, the effects of a diet containing juniper berry oil on hepatic reperfusion injury in a low-flow, reflow reperfusion model were investigated in the rat. Rats were fed semisynthetic diets containing either juniper berry oil, fish oil, or corn oil for 14 to 16 days. Daily food consumption averaged around 20 g/d in both the control and treatment groups; average daily weight gain was around 4 g per 100 g rat weight in all three groups studied, and there were no significant differences in these parameters. Livers were initially perfused at low-flow rates to induce pericentral hypoxia followed by a 40-minute reperfusion period. Peak lactate dehydrogenase (LDH) release during reflow averaged 44 U/g/h in the corn oil group and 32 U/g/h in the fish oil group, but was only 21 U/g/h as a result of juniper berry oil treatment. Malondialdehyde (MDA), an end-product of lipid peroxidation, reached a maximum value of 62 nmol/g/h in the corn oil group, but only reached 43 nmol/g/h and 34 nmol/g/h in the fish oil and juniper berry oil groups, respectively. Both juniper berry oil and fish oil treatment improved rates of bile flow from 25 μL/g/h (corn oil) to 36 and 38 μL/g/h, respectively. Importantly, juniper berry oil reduced cell death in pericentral regions of the liver lobule by 75%. Trypan blue distribution time, an indicator of the hepatic microcirculation, was reduced by approximately 25% with fish oil and over 50% by juniper berry oil diets compared with corn oil controls. The rates of entry of fluorescein-dextran, a dye confined to the vascular space, were increased 1.8- and 2.6-fold, and rates of outflow were increased 4.4- and 4.3-fold by fish oil and juniper berry oil, respectively, also reflecting improved microcirculation. Juniper berry oil also blunted increases in intracellular calcium and release of prostaglandin E_2 (PGE_2) by cultured Kupffer cells stimulated by endotoxin. These results are consistent with the hypothesis that feeding a diet containing juniper berry oil reduces reperfusion injury by inhibiting activation of Kupffer cells, thus reducing vasoactive eicosanoid release and improving the hepatic microcirculation in livers undergoing oxidant stress. (HEPATOLOGY 1998;28:1042-1050.)

Kupffer cells, the resident macrophages in the liver, are a major source of cytokines[1] and produce 70% to 80% of the eicosanoids from arachidonic acid in the liver.[2] Kupffer cells are activated by reperfusion after hypoxia,[3,4] and release toxic mediators such as proteases, tumor necrosis factors, and toxic free radicals[5-7] that participate in primary graft failure after liver transplantation. Excess production and release of arachidonic acid metabolites also occurs in pathophysiological states such as the reperfusion injury associated with cardiac arrhythmias, fatty liver in sepsis, inflammation, asthma, and arthritis.[8]

Polyunsaturated fatty acids in macrophage membranes serve as substrates for the formation of eicosanoid mediators. Arachidonic acid (20:4ω6), derived from dietary linoleic acid (18:2ω6) from sources such as corn oil, generates eicosanoids via the cyclooxygenase and lipo-oxygenase pathways.[9] In contrast, fish oil contains high levels of eicosapentaenoic acid (EPA) (20:5ω3) and docosahexaenoic acid (DHA) (22:6ω3). Dietary provision of fish oil polyunsaturated fatty acids modulates the fatty acid composition of cell membrane phospholipids by displacing 20:4ω6 and 18:2ω6. Eicosanoids derived from 20:5ω3 have reduced inflammatory properties compared with those derived from 20:4ω6.[10-12] Indeed, fish oil diets attenuated myocardial dysfunction and injury caused by global ischemia and reperfusion in isolated rat hearts,[13] prevented fatty infiltration of the liver induced by sepsis,[14] inhibited rejection episodes, improved renal hemodynamics and blood pressure in renal transplant recipients,[15] and improved cardiopulmonary response to bacteremia, including improved oxygenation and systemic perfusion.[16] In contrast, dietary fish oil exacerbated alcohol-induced liver damage for reasons that remain unclear.[17] Finally, fish oil prevented hypoxia-reoxygenation injury in the liver.[18] Reperfusion injury is a major cause of both alcoholic liver disease and graft failure following transplantation.[19,20]

Juniper berry oil, which is rich in 20:3[5,11,14] and 20:4,[5,11,14,17] is more competitive with arachidonate for incorporation into phospholipids than the EPA and DHA prevalent in fish oil, and therefore rapidly replaces arachidonate in membranes.[21,22] The fatty acid 20:3[5,11,14] is extensively incorporated into phospholipids in liver tissue, especially phosphatidylinositol. Like fish oil, the lipids found in juniper berry oil are also unable to form leukotrienes and prostaglandins, because they lack the delta-8 double bond. These oils may

Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; LDH, lactate dehydrogenase; MDA, malondialdehyde; $[Ca^{2+}]_i$, intracellular calcium; PGE_2, prostaglandin E_2; TXA_2, thromboxane A_2.

From the Laboratory of Hepatobiology and Toxicology, Department of Pharmacology, The University of North Carolina at Chapel Hill, Chapel Hill, NC.

Received December 17, 1997; accepted May 4, 1998.

Supported in part by grants from NIAAA (AA03624) and Novartis Nutrition.

Address reprint requests to: Dr. Ronald G. Thurman, Laboratory of Hepatobiology and Toxicology, Department of Pharmacology, CB #7365, Faculty Laboratory Office Building, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7365. Fax: (919) 966-1893.

0270-9139/98/2804-0019$3.00/0

TABLE 1. Diet Composition

	Corn Oil (%)	Fish Oil* (%)	Juniper Berry Oil (%)
Casein	15	15	15
Sucrose	51	40	51
Corn oil	5	0	0
Encapsulated fish oil	0	25	0
Juniper berry oil	0	0	5
Cellulose	5	5	5
Mineral mixture	3.5	3.5	3.5
Vitamin mixture	1	1	1
DL-Methionine	0.3	0.3	0.3
Choline bitartrate	0.2	0.2	0.2
Corn starch	15	10	15
Gum arabic	4	0	4

*Composition of fish oil diet alone and does not include the gum arabic used in encapsulation.

therefore have profound effects on numerous disease states.[21] For example, dietary treatment with these lipids has been shown to reduce levels of antierythrocyte antibodies and improve survival of NZB mice.[22] Accordingly, the purpose of this study was to test the hypothesis that juniper berry oil could prevent ischemia/reperfusion injury by improving microcirculation in the perfused rat liver.

MATERIALS AND METHODS

Materials. All diets were the kind gift of Novartis Nutrition (Minneapolis, MN); all chemicals were reagent grade from standard sources.

Animals. Male Sprague-Dawley rats were obtained from Zivic Miller Laboratories (Zelienope, PA) and weighed between 110 and 150 g. Animals were fed semisynthetic diets containing either corn oil (Columbus Foods, Chicago, IL), encapsulated fish oil (Omega Protein, Reedville, VA), or juniper berry oil (Hauser Chemical Research, Boulder, CO) in an *ad libitum* design for 14 to 16 days (see Table 1 for diet composition). All diets were packed in individual 100-g cans under nitrogen and stored at 4°C to prevent oxidation of oils. Fresh diet was given daily, and average daily food consumption was calculated by weighing the remaining diet. All animals in this study received humane care in compliance with institutional guidelines.

Liver Perfusion. Rats were fasted for 24 hours and subsequently anesthetized with phenobarbital (75 mg/kg) before surgery. Livers were perfused via cannulation of the portal vein with Krebs-Henseleit bicarbonate buffer (pH 7.4, 37°C) saturated with an oxygen–carbon dioxide (95:5) gaseous mixture in a nonrecirculating perfusion system. The flow rate was equal to 1 mL/g/min for the initial 75 minutes of perfusion (low-flow period) and was followed with a 40-minute period at normal flow rates (4 mL/g/min). During the first 75-minute low-flow period, pericentral regions of the liver lobule were anoxic, while pericentral regions remained normoxic. Oxygen concentration in the effluent perfusate was monitored using a Clark-type, Teflon-shielded oxygen electrode; oxygen uptake was determined from influent minus effluent perfusate oxygen concentration differences, the flow rate, and the liver wet weight.

TABLE 2. Average Daily Food Consumption and Weight Gain of Rats on Corn Oil, Fish Oil, and Juniper Berry Oil Diets

Diet Group	Average Daily Diet Consumption (g)	Average Daily Weight Gain (g/100 g rat)
Corn oil	22 ± 1	4.4 ± 0.2
Fish oil	18 ± 1	4.2 ± 0.2
Juniper Berry oil	20 ± 2	4.6 ± 0.2

NOTE. Values are expressed as means ± SEM with n = 6 to 12 in each group.

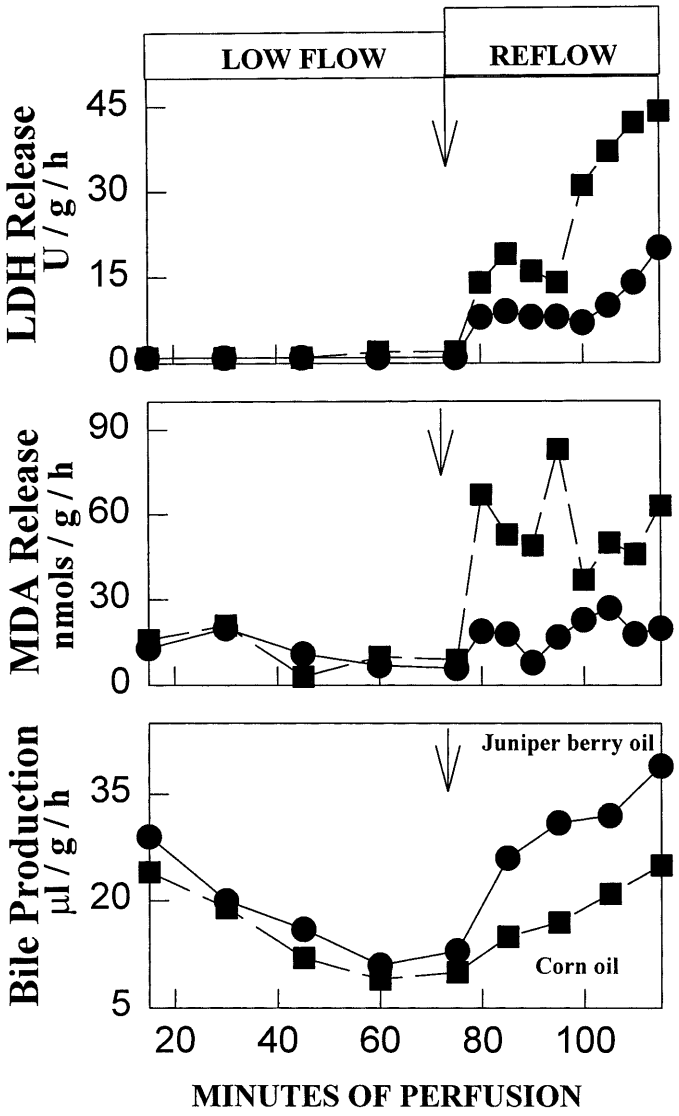

FIG. 1. Bile production, LDH, and MDA release in a typical low-flow, reflow liver perfusion. Livers from fasted rats were perfused at flow rates of 1 mL/g/min with Krebs-Henseleit buffer (pH 7.4, 37°C) in a nonrecirculating system. After 75 minutes, normal (4 mL/g/min) flow rates were restored for 40 minutes. LDH release into the effluent perfusate was determined using standard enzymatic techniques. The thiobarbituric acid method was used to measure MDA as described in Methods, and bile was collected via a cannula placed in the common bile duct. This graph represents a typical experiment for a liver from a rat fed a corn oil– (■) or juniper berry oil–containing (●) diet.

Bile Production. The common bile duct was cannulated with a segment of PE-10 tubing before liver perfusion. Aliquots of bile were taken at regular intervals throughout the perfusion with flow rates expressed in microliters per gram per hour.

Lactate Dehydrogenase and Malondialdehyde Determinations. Lactate dehydrogenase (LDH) assays were performed using standard enzymatic techniques.[23] Malondialdehyde (MDA) concentration in the effluent perfusate was determined using the thiobarbituric acid method as described elsewhere.[24] Rates of both LDH and MDA release were expressed per gram wet liver weight per hour.

Assessment of Microcirculation. Fluorescein isothiocyanate dextran, a dye confined to the vascular space,[25] was dissolved in perfusate at a concentration of 12 µmol/L and perfused into the liver for 2.5

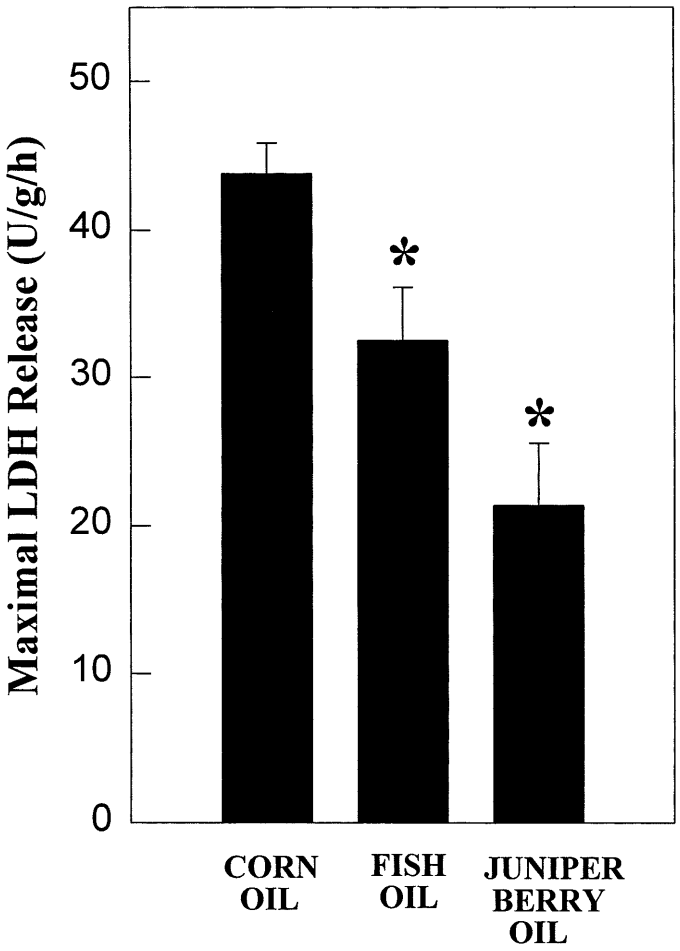

FIG. 2. Effects of corn oil, fish oil, and juniper berry oil on maximal LDH release. Rats were fed and livers were perfused as described in Methods. LDH activity in perfusate was determined with standard enzymatic methods.[23] Values are the mean of the maximal values of LDH release during 40 minutes of reperfusion ± SEM (n = 4-9 in each group; *$P < .05$, ANOVA).

minutes starting before the low-flow period or 40 minutes after reflow to assess hepatic microcirculation. Fluorescence of fluorescein-dextran was detected from the surface of the liver with a bifurcated-light guide system by placing the tip near the surface of the liver. One branch of the light guide was attached to a light source (mercury arc lamp, 430 nm), and the other branch was connected to a photomultiplier equipped with a secondary filter to remove light at wavelengths less than 515 nm (Wratten #16, Kodak, Rochester, NY). Fluorescein-dextran was infused for 2.5 mininutes, and slopes of fluorescein-dextran wash-in and wash-out were calculated from changes of surface fluorescence and presented as a percentage of basal fluorescence per unit time.

Trypan Blue Distribution Time and Histological Procedures. Trypan blue (0.2 mmol/L) was infused into the liver following each perfusion to assess cell death. The amount of time required to stain the liver exterior very dark blue was recorded to verify changes in microcirculation detected with fluorescein isothiocyanate dextran, followed by removal of excess dye with additional perfusate. Sections of fixed liver tissue were embedded in paraffin, counterstained with eosin, and processed for light microscopy. The nuclei of damaged cells only are positively stained with the dye, so that the percentage of cell death in the tissue may be determined.

Kupffer Cell Preparation and Culture. Kupffer cells were isolated by collagenase digestion and differential centrifugation using Percoll (Pharmacia, Uppsala, Sweden) as described elsewhere, with slight modifications.[26] Briefly, the liver was perfused through the portal vein with Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution at 37°C for 5 minutes at a flow rate of 26 mL/min. Subsequently, perfusion was with Hanks' balanced salt solution containing 0.025% collagenase IV (Sigma Chemical Co., St. Louis, MO) at 37°C for 5 minutes. After the liver was digested, it was excised and cut into small pieces in collagenase buffer. The suspension was filtered through nylon gauze mesh, and the filtrate was centrifuged at 450*g* for 10 minutes at 4°C. Cell pellets were resuspended in buffer, parenchymal cells were removed by centrifugation at 50*g* for 3 minutes, and the nonparenchymal cell fraction was washed twice with buffer. Cells were centrifuged on a density cushion of Percoll at 1,000*g* for 15 minutes, and the Kupffer cell fraction was collected and again washed with buffer. Viability of cells determined by Trypan blue exclusion was >90%, and purity was >85%. Cells were seeded onto 25-mm glass coverslips for intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurements or seeded onto 60-mm culture dishes to assess prostaglandin E_2 (PGE_2) production. Cells were cultured in 3 mL RPMI 1640 (GIBCO Laboratories Lite Technologies Inc., Grand Island, NY), supplemented with 10% fetal bovine serum, 10 mmol/L HEPES, and antibiotics (100 U/mL of penicillin G and 100 µg/mL of streptomycin sulfate) at 37°C with 5% CO_2. Nonadherent cells were removed after 1 hour by replacing buffer, and cells were cultured for 24 hours before $[Ca^{2+}]_i$ measurements or 6 hours for PGE_2 determinations.

Measurement of $[Ca^{2+}]_i$. Intracellular calcium was measured fluorometrically using the fluorescent calcium indicator dye, fura-2, and a microspectrofluorometer (PTI, South Brunswick, NJ) interfaced with an inverted microscope (Nikon, Tokyo, Japan). Kupffer cells

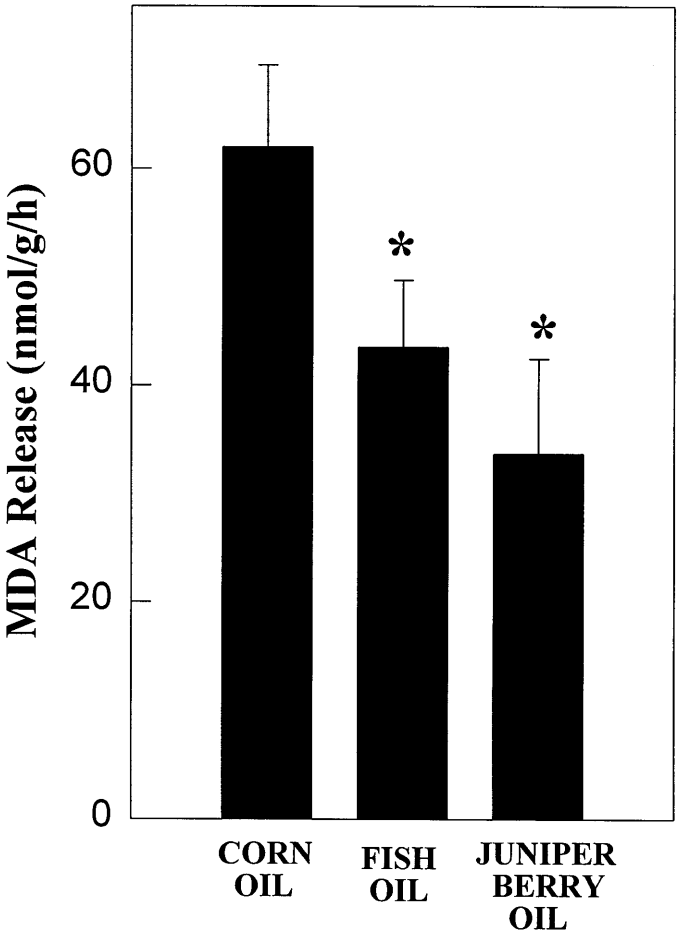

FIG. 3. Effects of dietary corn oil, fish oil, and juniper berry oil on MDA production. Conditions as in Fig. 1. MDA in perfusate was assessed using the thiobarbituric acid method. Values are the mean of the maximal rates of MDA production during the reperfusion period ± SEM (n = 4 in each group; *$P <$ .05, ANOVA).

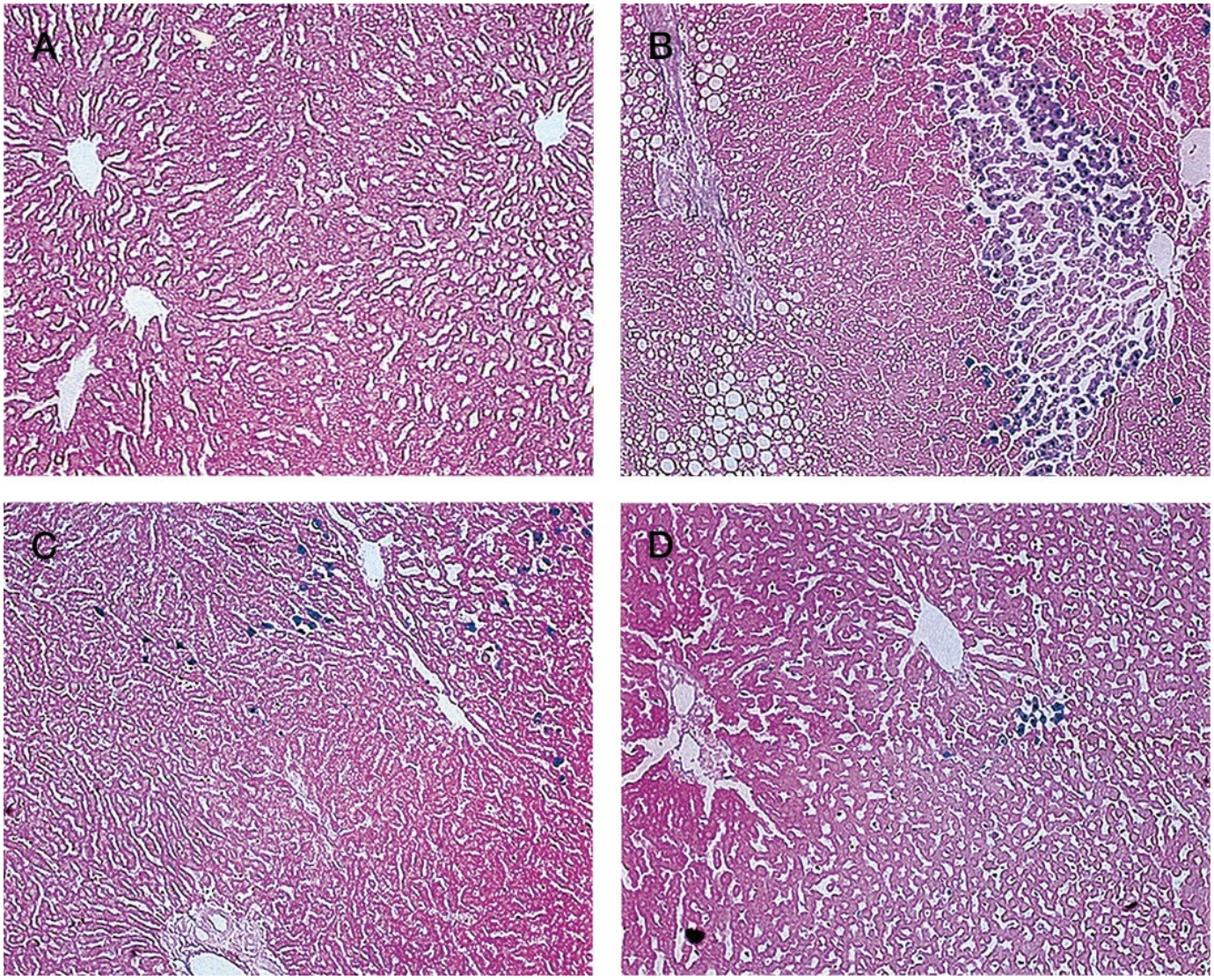

FIG. 4. Effects of corn oil, fish oil, and juniper berry oil on cell death. Trypan blue (0.2 mmol/L) was infused into livers following perfusion, and sections of liver tissue were processed for light microscopy. Representative images of at least four sections are shown. (Original magnification ×100.) (A) Liver tissue from a corn oil–treated rat before low-flow. (B) Liver tissue from a corn oil–treated rat after 40 minutes of reflow. (C) Liver tissue from a fish oil–treated rat after 40 minutes of reperfusion. (D) Liver tissue from a juniper berry oil–treated rat after reperfusion.

were incubated in modified Hanks' buffer (115 mmol/L NaCl, 5 mmol/L KCl, 0.3 mmol/L Na_2HPO_4, 0.4 mmol/L KH_2PO_4, 5.6 mmol/L glucose, 0.8 mmol/L $MgSO_4$, 1.26 mmol/L $CaCl_2$, 15 mmol/L HEPES [pH 7.4]) containing 5 μmol/L fura-2 acetoxymetyl ester (Molecular Probes Inc., Eugene, OR) and 0.03% Pluronic F127 (BASF Wyandotte, Wyandotte, MI) at room temperature for 60 minutes. Coverslips plated with Kupffer cells were rinsed and placed in chambers with buffer at room temperature. Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 nm and 380 nm and emission at 510 nm were monitored in individual Kupffer cells. Each value was corrected by subtracting the system dark noise and autofluorescence, assessed by quenching fura-2 fluorescence with Mn^{2+}, as described previously. Intracellular calcium was determined from the equation: $[Ca^{2+}]_i = K_d[(R - R_{min})/(R_{max} - R)]/(Fo/Fs)$, where Fo/Fs is the ratio of fluorescent intensities evoked by 380-nm light from fura-2 pentapotassium salt loaded in cells using a buffer containing 3 mmol/L ethylene glycol bis-(β-aminoethyl ether)-*N*,*N*-tetraacetic acid and 1 μmol/L ionomycin ($[Ca^{2+}]_{min}$) or 10 mmol/L Ca^{2+} and 1 μmol/L ionomycin ($[Ca^{2+}]_{max}$). R is the ratio of fluorescent intensities at excitation wavelengths of 340 nm and 380 nm, and R_{max} and R_{min} are values of R at $[Ca^{2+}]_{max}$ and $[Ca^{2+}]_{min}$, respectively. The values of these constants were determined at the end of each experiment, and a dissociation constant of 135 nmol/L was used.[27]

Measurement of PGE_2 in Conditioned Media From Cultured Kupffer Cells. Kupffer cells isolated from rats were kept in primary culture containing 10 μg/mL endotoxin for 6 hours. Supernatants were collected and analyzed for PGE_2 by competitive radioimmunoassay using ^{125}I-labeled PGE_2 from Advanced Magnetics (Cambridge, MA).

Statistical Analysis. The Student's *t* test or ANOVA plus the Student Newman-Keuls post-hoc test were used where appropriate. Differences were considered significant at $P < .05$.

RESULTS

Diet Consumption and Weight Gain. Semisynthetic diets containing 5% (wt/wt) corn oil, fish oil, or juniper berry oil were fed for a 14- to 16-day period. The average daily food consumption throughout the feeding period was around 20 g per rat per day in all three groups (Table 2). Each rat was weighed daily for the duration of the feeding period; the average daily weight gain per 100 g total body weight was

around 4 g/d in all three groups (Table 2). There were no significant differences in these parameters.

Effects of Fish Oil and Juniper Berry Oil on Liver Injury. LDH release before and during the low-flow period was negligible (approximately 2-4 U/g/h) in all groups studied (data not shown). Upon reflow, an increase in LDH activity was observed in all three groups, reaching a maximal value of 44 U/g/h in the corn oil control group (Fig. 1). Treatment with fish oil blunted the increase in LDH release to 32 U/g/h, whereas juniper berry oil feeding significantly minimized the LDH peak release during reflow with values only reaching 21 U/g/h (n = 4-9; $P < .05$) (Fig. 2).

Before and during the low-flow period, MDA release remained below 10 nmol/g/h in all three groups studied (data not shown). MDA concentrations in the effluent during reflow increased and reached a new maximal value of 62 ± 15 nmol/g/h in the corn oil group (Fig. 3). Maximal MDA production was minimized by both fish and juniper berry oil treatments, only reaching values of 42 ± 11 nmol/g/h and 34 ± 17 nmol/g/h, respectively (n = 4-5; $P = .05$) (Fig. 3).

The histology from some corn oil–fed rats showed evidence of steatosis, and this phenomenon was less overt in fish oil– or juniper berry oil–fed rats. However, no irreversible cell injury was observed before hypoxia-reperfusion in all groups studied. Irreversible cellular injury in pericentral regions of the liver lobule indicated by nuclear Trypan blue uptake occurred after 40 minutes of reperfusion (Fig. 4B). Treatment with either fish oil or juniper berry oil significantly reduced fat accumulation as well as the number of positively stained cells, verifying the cytoprotective effect of these diets (n = 4; $P < .05$) (Figs. 4C, 4D, Fig. 5).

Hepatic Microcirculation. Trypan blue distribution time, an index of the hepatic microcirculation, was reduced significantly by approximately 25% with fish oil treatment and by nearly 50% with juniper berry oil when compared with the corn oil control (n = 4-7; $P < .05$) (Fig. 6).

Fluorescence-dextran, a dye confined to the vascular space, was infused into the liver for 2.5 minutes starting before the low-flow period or 40 minutes after reflow to assess hepatic microcirculation.[25] Surface fluorescence (430 → 550 nm) increased gradually upon fluorescein-dextran infusion into the liver, and reached a steady state in about 1 minute (Fig. 7). After infusion of fluorescein-dextran was terminated, surface fluorescence gradually returned to basal levels. The rates of fluorescein-dextran entry and exit into the vascular space were determined by measuring the slopes of surface fluorescence increase upon infusion (wash-in) and decrease after termination (wash-out). Rates of fluorescein wash-in were 6.3 (change as percent of basal/time) and wash-out 4.8 before the low-flow period (data not shown). These rates were similar in all groups studied. Rates of fluorescein wash-in were decreased by 60% and wash-out by 73% after 40 minutes of reperfusion in corn oil–fed rats. Dietary fish oil and juniper berry oil largely blocked these alterations (Figs. 7 and 8), demonstrating that these diets indeed dramatically improve hepatic microcirculation upon reflow (Fig. 8).

Bile Production. Flow rates of bile slowly tapered off during the low-flow period, reaching minimum values of approximately 8 μL/g/h. Upon reflow, however, bile production increased gradually, reaching a new maximal value of 28 μL/g/h in the corn oil group. Both fish oil and juniper berry oil substantially improved rates of bile flow upon reflow to 36 ± 5 μL/g/h and 38 ± 2 μL/g/h, respectively (n = 4-6) (Fig. 9).

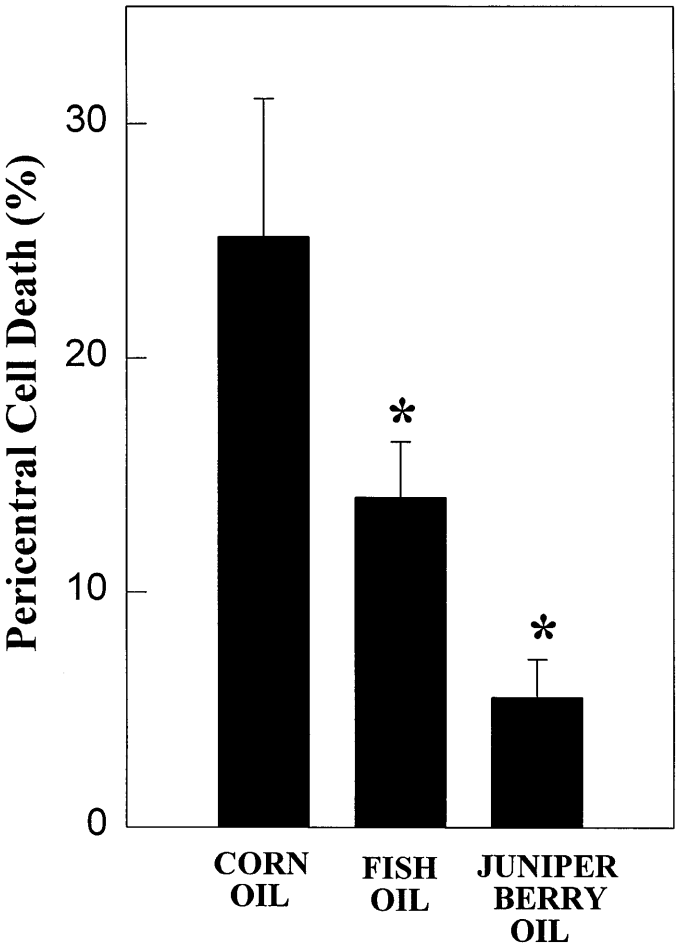

FIG. 5. Effects of corn oil, fish oil, and juniper berry oil on cell death in pericentral regions of the liver lobule. Livers were perfused as described in Methods. Trypan blue was infused into the liver at the end of 40 minutes of reperfusion at a final concentration of 0.2 mmol/L. All nuclei of parenchymal cells in a zone radiating five cells from pericentral regions were identified as Trypan blue–positive or –negative. The percentage of staining was calculated from the number of stained nuclei divided by the total number of cells in any given region. Values are means ± SEM (n = 4 in each group; $*P < .05$, ANOVA).

Effects of Juniper Berry Oil on $[Ca^{2+}]_i$ Levels and Release of PGE_2 by Kupffer Cells. Intracellular calcium was monitored fluorometrically in cultured Kupffer cells (Fig. 10A). After addition of lipopolysaccharide (10 μg/mL), $[Ca^{2+}]_i$ increased rapidly and reached values around 260 nmol/L in Kupffer cells isolated from rats fed the corn oil control diet. Juniper berry oil significantly blunted the increase in $[Ca^{2+}]_i$, indicating that it prevents lipopolysaccharide-induced activation of Kupffer cells.

Kupffer cells are the major source of eicosanoids in the liver.[2] Therefore, production of PGE_2, a vasoactive eicosanoid,[28] was measured in cultured Kupffer cells. After lipopolysaccharide (10 μg/mL) stimulation, PGE_2 levels in culture media increased by 152% (Fig. 10B) in Kupffer cells from corn oil control rats; however, in cells from juniper berry oil–treated rats, the increase was blunted significantly. Thus, juniper berry oil minimizes PGE_2 production by Kupffer cells.

DISCUSSION

Juniper Berry Oil Minimizes Hepatic Reperfusion Injury. Previous studies have demonstrated protective effects of fish oil

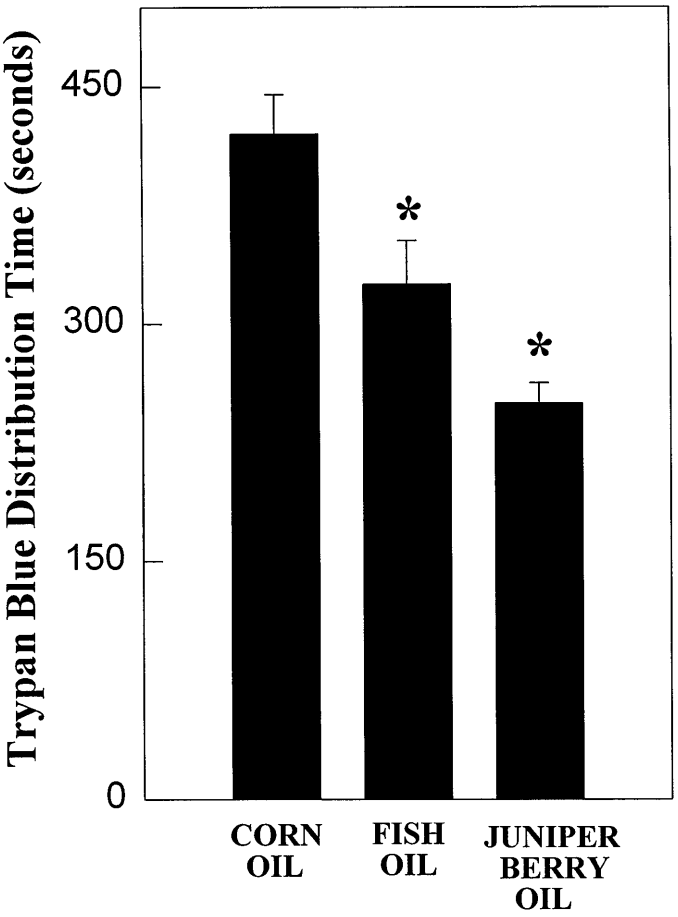

FIG. 6. Effects of corn oil, fish oil, and juniper berry oil treatment on Trypan blue distribution time. Trypan blue was infused into the liver at final concentrations of 0.2 mmol/L at the end of a 40-minute reperfusion period, and the time for Trypan blue to distribute evenly on the liver surface was recorded. Values are means ± SEM (n = 4-7 in each group; *$P < .05$, ANOVA).

against tissue damage in ischemic-reperfused rat heart, fatty liver caused by sepsis, rejection after renal transplantation, and cardiopulmonary responses to bacteremia.[13-16] These effects were presumably caused by replacement of arachidonic acid with EPA and DHA.[13-16] However, inflammation and necrosis in the liver were increased in rats fed ethanol and fish oil compared with corn oil, and focal fibrosis was predominant in rats fed ethanol and fish oil.[17] Thus, fish oil enhanced some of the deleterious effects of ethanol, which could be caused by increased substrate for lipid peroxidation. Feeding-peroxidized fish oil could also lead to an increase in lipid metabolites, thus confounding interpretation. Indeed, fish oil enhanced lipid peroxidation in cultured hepatocytes, in animals, and in a variety of clinical settings.[29-32] In this study, the effects of fish oil and juniper berry oil on hepatic reperfusion injury were compared using a low-flow, reflow model, in which Kupffer cells, the major source of eicosanoids in the liver,[1] are activated.[3,4] In this model, flow rates are reduced during the initial 75 minutes of the perfusion to induce pericentral hypoxia. Subsequently, normal flow rates are restored, producing an oxygen-dependent reperfusion injury in pericentral regions of the liver lobule. To avoid oxidation of fish oil, it was encapsulated and diets were stored cold under a nitrogen atmosphere. This precaution was taken because feeding unencapsulated fish oil led to liver injury.[17] Under such circumstances, livers from rats fed fish and juniper berry oil–containing diets released significantly less LDH during reperfusion compared with rats fed corn oil–containing diets (Fig. 2). MDA release followed a similar pattern (Fig. 3). Additionally, both juniper berry oil and fish oil significantly reduced cell death detected with Trypan blue by over 75% and 50%, respectively, indicating that both oils were cytoprotective in this model of hypoxia-reoxygenation (Figs. 4 and 5). Moreover, hepatic reperfusion injury was reduced by juniper berry oil to an even greater extent than by fish oil.

Role of Microcirculation in the Protective Effect of Fish Oil and Juniper Berry Oil. Previous studies have shown that changes in microcirculation can lead to hepatic reperfusion injury. Moreover, improving the microcirculation with either arginine or glycine reduces reperfusion injury in this model.[24,33] Alcohol increased portal pressure and hepatic reperfusion injury concomitantly,[34] and alteration of the hepatic microcirculation that occurs after liver transplantation was associated

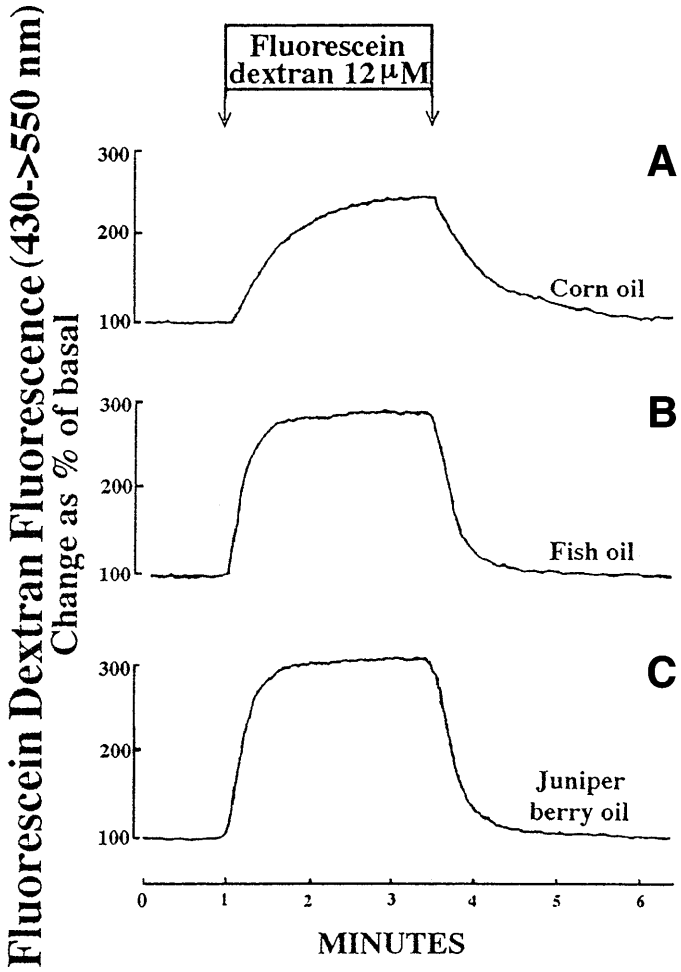

FIG. 7. Representative traces of fluorescence of fluorescein isothiocyanate dextran on the surface of perfused livers from corn oil–, fish oil–, and juniper berry oil–treated rats. Fluorescein isothiocyanate dextran was dissolved in perfusate at a concentration of 12 μmol/L and perfused into the liver for 2.5 minutes starting at 40 minutes after reflow. Fluorescence of fluorescein-dextran (430 → 520 nm) was detected from the surface of the liver with a bifurcated-light guide system by placing the tip near the surface of the liver as described in Methods. Representative traces of fluorescence from four to five livers per group are shown.

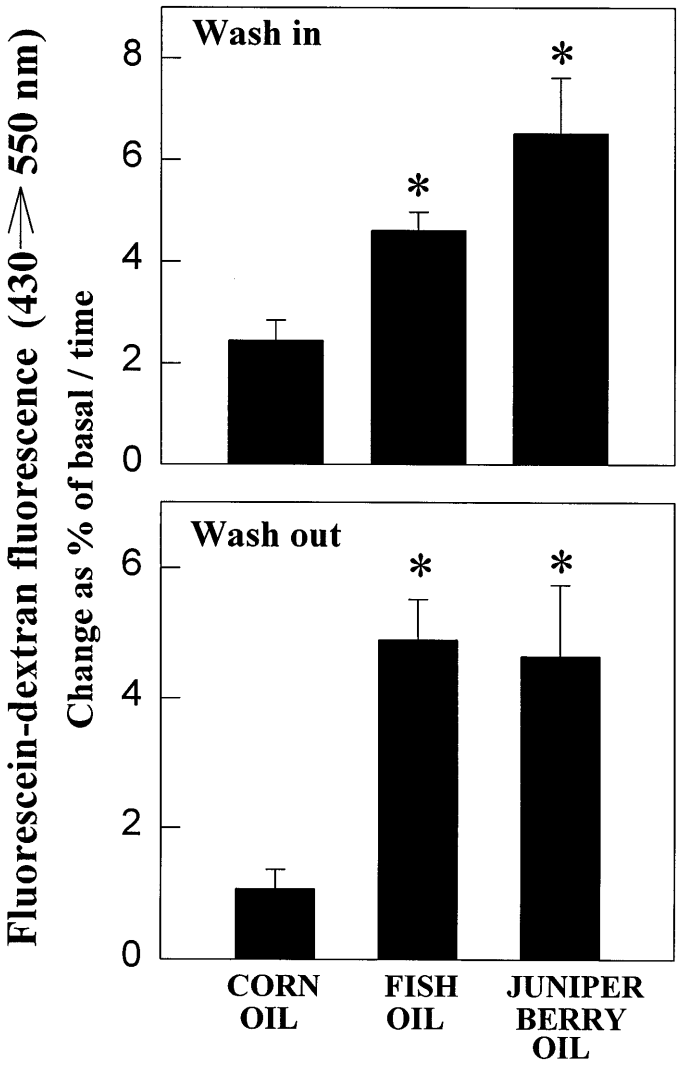

FIG. 8. Effects of corn oil, fish oil, and juniper berry oil on fluorescene of fluorescein isothiocyanate dextran on the surface of perfused livers. Conditions as in Fig. 7. Fluorescein-dextran was infused for 2.5 minutes, and slopes of fluorescein-dextran entry (wash-in) and exit (wash-out) were calculated from changes of surface fluorescence as a percentage of basal values per unit time. Values are means ± SEM (n = 4-5 in each group). *$P <$ .05 compared with corn oil–treated groups.

with primary graft failure.[35] Eicosanoids such as PGF_2 and thromboxane A_2 (TXA_2) have been found to stimulate contractility of hepatic stellate cells,[36] and PGE_2, PGD_2, PGF_2, and TXA_2 released from Kupffer cells in the intact organ increased portal pressure and decreased perfusion flow in the isolated perfused rat liver.[28,36] Furthermore, analogues of TXA_2 also increased perfusion pressure.[36] Fish oil and juniper berry oil most likely decrease production of vasoactive eicosanoids, thus improving hepatic microcirculation.[18] Indeed, the time required for Trypan blue to distribute evenly in the liver, which depends mainly on hepatic microcirculation, was much shorter in the fish oil– and juniper berry oil–treated groups than in the corn oil–treated control group (Fig. 6). Moreover, bile production, which is related to energy supply, which in turn is regulated by oxygen delivery via the microcirculation, was improved by both fish oil and juniper berry oil (Fig. 9). Fluorescein isothiocyanate dextran is a dye confined to the vascular space. Previous studies have proven that fluorescence of fluorescein-dextran detected on the liver surface is a sensitive indicator of hepatic microcirculation.[24,25,33] Here, rates of fluorescein-dextran entry and exit into the vascular space of the liver were increased significantly in livers from fish oil– and juniper berry oil–treated compared with corn oil–treated rats after reperfusion (Fig. 8), confirming that fish oil and juniper berry oil improved hepatic microcirculation as was suggested by data with Trypan blue distribution time and bile production. Taken together, it is concluded that fish oil and juniper berry oil minimize hepatic reperfusion injury by improving the hepatic microcirculation.

How does juniper berry oil improve hepatic microcirculation? It is well known that Kupffer cells are the major source of eicosanoids in the liver.[2] Arachidonic acid derived from linoleic acid in corn oil serves as a substrate for the formation of eicosanoid mediators, and many of these mediators are known to be vasoactive, such as PGE_2, PGD_2, $PGF_{2\alpha}$, TXA_2, and leukotriene B_4. Dietary fish oil and juniper berry oil, which are rich in EPA and DHA, displace linoleic acid and arachidonic acid, thus reducing eicosanoids derived from

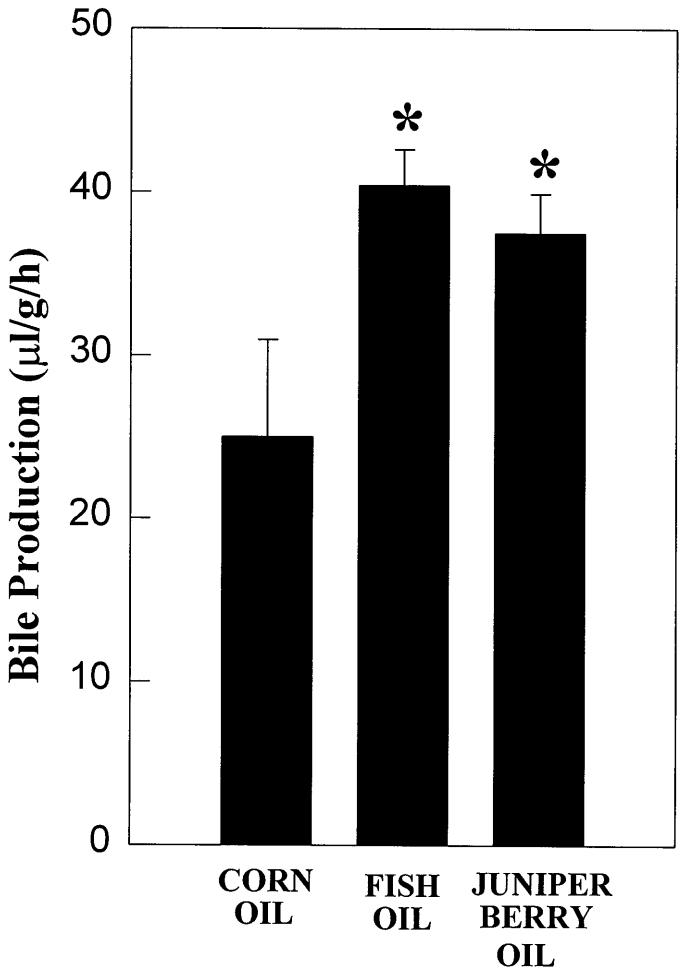

FIG. 9. Effects of corn oil, fish oil, and juniper berry oil treatment on maximal rates of bile production. Conditions as in Fig. 1. The common bile duct was cannulated with a segment of PE-10 tubing before liver perfusion. Aliquots of bile were taken at regular intervals and mean flow rates over 40 minutes of the reperfusion period are expressed in microliters per gram per hour. Values are means ± SEM (n = 2-6 in each group). *$P <$ .05 compared with corn oil–treated groups.

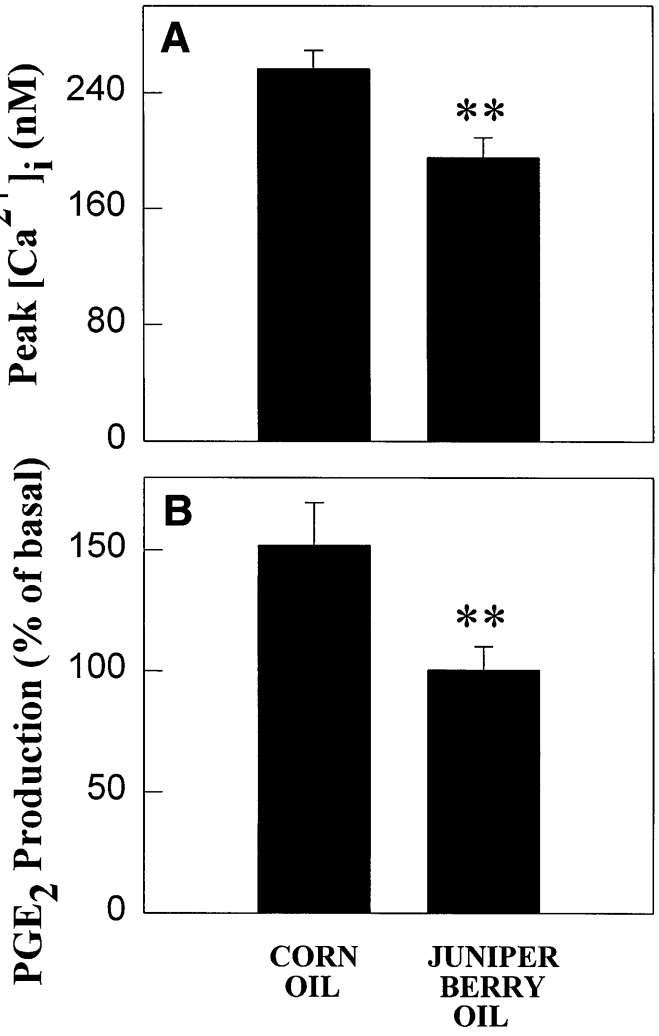

FIG. 10. Effects of juniper berry oil on intracellular calcium and PGE_2 production in cultured Kupffer cells. Rats were fed diets containing corn oil or juniper berry oil as described in Methods. Kupffer cells were isolated by collagenase digestion and differential centrifugation using Percoll[26] and cultured in RPMI 1640 for 24 hours before $[Ca^{2+}]_i$ measurement (A) or 6 hours for PGE_2 determination (B). $[Ca^{2+}]_i$ was measured fluorometrically using the calcium indicator dye, fura-2, and a microspectrofluorometer interfaced with an inverted microscope. Changes in fluorescence intensity of fura-2 at excitation wavelengths of 340 nm and 380 nm and emission at 510 nm were monitored in individual Kupffer cells. Each value was corrected by subtracting the system dark noise and autofluorescence, assessed by quenching fura-2 fluorescence with Mn^{2+}.[27] PGE_2 in the culture media was determined by competitive radioimmunoassay using ^{125}I-labeled PGE_2 from Advanced Magnetics). Values are means ± SEM (n = 5-11 in each group). *$P < .05$ compared with corn oil–treated groups (Student's *t* test).

20:4ω6.[9,21,22] Furthermore, juniper berry oil has been shown to replace arachidonic acid more rapidly than fish oil.[21,22] Previous studies including dietary provision for 2 to 6 weeks or enteral feeding of fish oil for 4 days or culture of Kupffer cells with EPA or DHA significantly reduced 18:2ω6 and 20:4ω6, increased 20:5ω3 components in cell membrane phospholipids in Kupffer cells, and decreased production of arachidonic acid–derived eicosanoids such as PGE_2, TXA_2, and TXB_2.[9,37,38] Eicosanoids derived from 20:5ω3 have diminished inflammatory and vasoactive properties.[8,10-12] Moreover, fish oil inhibits Δ-6 desaturase and cyclo-oxygenase, thereby reducing the metabolism of arachidonic acid and production of eicosanoids.[9] By preventing the formation of deleterious eicosanoids, fish oil treatment also decreases vasoconstriction and subsequent microcirculatory failure. In support of this idea, it was demonstrated here that juniper berry oil prevented activation of Kupffer cells and minimized production of the vasoactive eicosanoid, PGE_2 (Fig. 10). This information leads to the conclusion that juniper berry oil improves hepatic microcirculation by reducing the production of vasoactive eicosanoids by Kupffer cells.

Taken together, these findings indicate that juniper berry oil treatment improved hepatic microcirculation to an even greater extent than fish oil, thus protecting against reperfusion injury more effectively. This result is most likely related to reduced production of arachidonic-derived vasoactive mediators. Reperfusion injury is considered a major cause of early graft damage affecting the outcome in liver transplantation surgery.[19] It is also a major cause of hepatic damage in alcohol-induced liver disease.[34] Therefore, improving the microcirculation with juniper berry oil may be an effective way to minimize reperfusion injury, thereby serving as a useful therapy in numerous clinical situations involving oxidative stress and inflammation.

REFERENCES

1. Decker K. Biologically active products of stimulated liver macrophages (Kupffer cells). Eur J Biochem 1990;192:245-261.
2. Altin JG, Bygrave FL. Non-parenchymal cells as mediators of physiological responses in liver. Mol Cell Biochem 1988;83:3-14.
3. Caldwell-Kenkel JC, Currin RT, Tanaka Y, Thurman RG, Lemasters JJ. Kupffer cell activation and endothelial cell damage after storage of rat livers: effects of reperfusion. HEPATOLOGY 1991;13:83-95.
4. Lindert KA, Caldwell-Kenkel JC, Nukina S, Lemasters JJ, Thurman RG. Activation of Kupffer cells on reperfusion following hypoxia: particle phagocytosis in a low-flow, reflow model. Am J Physiol 1992;262:G345-G350.
5. Bouwens L. Structural and functional aspects of Kupffer cells. Rev Biol Cell 1988;16:69-94.
6. Cerra FB, West MA, Keller G, Mazuski J, Simmons RL. Hypermetabolism/organ failure: the role of the activated macrophage as a metabolic regulator. Prog Clin Biol Res 1988;264:27-42.
7. Wardle EN. Kupffer cells and their function. Liver 1987;7:63-75.
8. Nakamura N, Hamazaki T, Yamazaki K, Taki H, Kobayashi M, Yazawa K, Ibuki F. Intravenous infusion of tridocosahexaenoyl-glycerol emulsion into rabbits: effects on leukotriene $B_{4/5}$ production and fatty acid composition of plasma and leukocytes. J Clin Invest 1993;92:1253-1261.
9. Palombo JD, Bistrian BR, Fechner KD, Blackburn GL, Forse RA. Rapid incorporation of fish or olive oil fatty acids into rat hepatic sinusoidal cell phospholipids after continuous enteral feeding during endotoxemia. Am J Clin Nutr 1993;57:643-649.
10. Terano T, Salmon JA, Moncada S. Effect of orally administered eisosapentaenoic acid (EPA) on the formation of leukotriene B_4 and leukotriene B_5 by rat leukocytes. Biochem Pharmacol 1984;33:3071-3076.
11. Lee TH, Hoover RL, Williams JD, Sperling RI, Ravalese J, Spur BW, Robinson DR, et al. Effect of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. N Engl J Med 1985;312:1217-1224.
12. Siess W, Scherer B, Bohlig B, Roth P, Kurzmann I, Weber PC. Platelet-membrane fatty acids, platelet aggregation, and thromboxane formation during a mackerel diet. Lancet 1980;1:441-444.
13. Yang B, Saldeen TGP, Nichols WW, Mehta JL. Dietary fish oil supplementation attenuates myocardial dysfunction and injury caused by global ischemia and reperfusion in isolated rat hearts. J Nutr 1993;2067-2074.
14. Lanza-Jacoby S, Smythe C, Phetteplace H, Tabares A. Adaptation to a fish oil diet before inducing sepsis in rats prevents fatty infiltration of the liver. J Parenteral Enteral Nutr 1992;16:353-358.
15. van der Heide JJH, Bilo HJG, Donker JM, Wilmink JM, Tegzess AM. Effect of dietary fish oil on renal function and rejection in cyclosporine-treated recipients of renal transplants. N Engl J Med 1993;329:769-773.
16. Murray MJ, Svingen BA, Holman RT, Yaksh TL. Effects of a fish oil diet on pigs' cardiopulmonary response to bacteremia. J Parenteral Enteral Nutr 1991;15:152-158.

17. Nanji AA, Zhao S, Sadrzadeh SM, Dannenberg AJ, Tahan SR, Waxman DJ. Markedly enhanced cytochrome P450 2E1 induction and lipid peroxidation is associated with severe liver injury in fish oil–ethanol–fed rats. Alcohol Clin Exp Res 1994;18:1280-1285.
18. Zhong Z, Thurman RG. A fish oil diet minimizes hepatic reperfusion injury in the low-flow, reflow liver perfusion model. HEPATOLOGY 1995;22:929-935.
19. Thurman RG, Marzi I, Seitz G, Thies J, Lemasters JJ, Zimmermann FA. Hepatic reperfusion injury following orthotopic liver transplantation in the rat. Transplantation 1988;46:502-506.
20. Zhong Z, Connor H, Mason RP, Qu W, Stachlewitz RF, Gao W, Lemasters JJ, et al. Destruction of Kupffer cells increases survival and reduces graft injury after transplantation of fatty livers from ethanol-treated rats. Liver Transplant Surg 1996;2:383-387.
21. Berger A, Fenz R, German JB. Incorporation of dietary 5,11,14-icosatrienoate into various mouse phospholipid classes and tissues. J Nutr Biochem 1996;4:409-420.
22. Lai LTY, Naiki M, Yoshida SH, German JBaMEG. Dietary *Plactycladus orientalis* seed oil suppresses anti-erythrocyte autoantibodies and prolongs survival of NZB mice. Clin Immunol Immunopathol 1996;71:293-302.
23. Bergmeyer, HU (ed). Methods of Enzymatic Analysis. New York: Academic, 1988.
24. Zhong Z, Jones S, Thurman RG. Glycine minimizes reperfusion injury in a low-flow, reflow liver perfusion model in the rat. Am J Physiol 1996;270:G332-G338.
25. Conway JG, Popp JA, Thurman RG. Microcirculation in periportal and pericentral regions of lobule in perfused rat liver. Am J Physiol 1985;249:G449-G456.
26. Pertoft H, Smedsrod B. Separation and characterization of liver cells. In: Pretlow II TG, Pretlow TP. Cell Separation: Methods and Selected Applications, Vol 4. New York: Academic, pp 1-24, 1987.
27. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260: 3440-3450.
28. Iwai M, Gardemann A, Püschel G, Jungermann K. Potential role for prostaglandin F_{2a}, D_2, E_2 and thromboxane A_2 in mediating the metabolic and hemodynamic actions of sympathetic nerves in perfused rat liver. Eur J Biochem 1988;175:45-50.
29. Reddy ACP, Lokesh BR. Alterations in lipid peroxides in rat liver by dietary n-3 fatty acids: modulation of antioxidant enzymes by curcumin, eugenol, and vitamin E. J Nutr Biochem 1994;5:181-188.
30. Sugihara N, Tsuruta Y, Date Y, Furuno K, Kohashi K. High peroxidative susceptibility of fish oil polyunsaturated fatty acid in cultured rat hepatocytes. Toxicol Appl Pharmacol 1994;126:124-128.
31. French SW. Nutrition in the pathogenesis of alcoholic liver disease. Alcohol Alcohol 1993;28:97-109.
32. Nanji AA, French SW. Effect of different dietary fats on ethanol metabolism: implications for pathogenesis of experimental alcoholic liver disease. Int J Vit Nutr Res 1988;58:475-476.
33. Jones SM, Thurman RG. L-Arginine minimizes reperfusion injury in a low-flow, reflow model of liver perfusion. HEPATOLOGY 1996;24:163-168.
34. Zhong Z, Connor HD, Mason RP, Qu W, Gao W, Lemasters JJ, Thurman RG. Role of Kupffer cells in reperfusion injury in fat-loaded livers from ethanol-treated rats. J Pharmacol Exp Ther 1995;27:1512-1517.
35. Marzi I, Takei Y, Knee J, Menger M, Gores GJ, Bühren V, Trentz O, et al. Assessment of reperfusion injury by intravital fluorescence microscopy following liver transplantation in the rat. Transplant Proc 1990;22:2004-2005.
36. Kawada N, Klein H, Decker K. Eicosanoid-mediated contractility of hepatic stellate cells. Biochem J 1992;285:367-371.
37. Ogle CK, Wu J-Z, Fischer JE, Alexander JW. The direct effect of eicosapentaenoic acid and desapentaenoic acid on the production of PGE_2 and TxB_2 by Kupffer cells. J Parenteral Enteral Nutr 1992;16:20S.
38. Billiar TR, Bankey PE, Svingen BA, Curran RD, West MA, Holman RT, Cerra FB. Fatty acid intake and Kupffer cell function: fish oil alters eicosanoid and monokine production to endotoxin stimulation. Surgery 1988;104:343-349.