

# Effects of Docosahexaenoic Acid in an Experimental Rat Model of Nonalcoholic Steatohepatitis

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**Abstract:** Docosahexaenoic acid (DHA) regulates the lipid metabolism and inflammation that is closely associated with oxidative stress. The present study investigated the effects of DHA on the development of nonalcoholic steatohepatitis (NASH). To induce fatty liver, rats were fed choline-deficient high-fat diets (CDHF). The rats were then divided into 4 groups treated over the subsequent 6 weeks as follows: control, CDHF, CDHF + oxidative stress (NASH), and NASH + DHA (1.0 g/kg, p.o.). Rats of the control group were fed MF chow diet only. NASH rats showed severe steatohepatitis and liver fibrosis. Treatment with DHA significantly decreased the n-6/n-3 ratio in the livers and increased plasma SOD like activity compared with NASH rats. In addition, DHA attenuated the liver fibrosis during NASH development. Therefore, a higher DHA ratio in the liver of NASH rats might regulate the inflammatory response through a low n-6 ratio and diminished oxidative stress, effectively inhibiting liver fibrosis during NASH progression. These results suggested that DHA is a novel functional food for the prevention of NASH.

**Key words:** docosahexaenoic acid, nonalcoholic steatohepatitis, n-6/n-3 ratio, hepatoprotection, DHA, liver fibrosis, oxidative stress, functional foods.

## 1 INTRODUCTION

The "two-hit theory" provides the most widely accepted explanation for the progression of nonalcoholic steatohepatitis (NASH)<sup>1)</sup>. This theory postulates that fat accumulation in the liver by itself is not detrimental, but rather, secondary insults imposed upon a fatty liver, such as those resulting from reactive oxygen species and inflammatory cytokines, are necessary for progression to steatohepatitis. In the previous study, we developed an animal model of NASH (Patent application No. PCT/JP2007/52477), and more recently, found that experimental NASH rats induced by intermittent administration of a sodium nitrate compound showed increased production of mitochondrial reactive oxygen species and decreased plasma antioxidant activities<sup>2)</sup>. In addition, we demonstrated that fermented green tea ex-

tracts<sup>3)</sup> and *Vitis coignetiae Pulliat* leaves<sup>4)</sup> prevented the progression of NASH, suggesting the possible involvement of oxidative stress as a second hit in the two-hit model of NASH progression.

Recently, it is reported that the fatty acid composition of the liver can influence the degree of liver injury and disease progression. NASH and nonalcoholic fatty liver disease (NAFLD) associated with obesity were linked to the depletion of hepatic n-3 long-chain polyunsaturated fatty acids (n-3 PUFAs)<sup>5)</sup>. The n-6/n-3 polyunsaturated fatty acid ratios of both steatosis and steatohepatitis patients are increased compared with healthy individuals<sup>6)</sup>. Mensink *et al.* reported that the decreasing total fat intake and increasing the intake of fish oils may be beneficial in the treatment of NASH<sup>7)</sup>. On the other hand, Allard *et al.* showed that

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Accepted February 4, 2010 (received for review November 17, 2009)

Journal of Oleo Science ISSN 1345-8957 print / ISSN 1347-3352 online  
<http://www.jstage.jst.go.jp/browse/jos/>

NASH patients have more metabolic abnormalities, but not the influence of dietary fatty acid composition<sup>8)</sup>. Thus, at present, there are various opinions whether increasing the intake of n-3 PUFA is effective on NASH development.

Docosahexaenoic acid (DHA) is the predominant n-3 PUFAs in marine fish. Dietary supplementation with DHA is effective for diseases such as hyperlipidemia, hypertension, atherosclerosis, and diabetes mellitus<sup>9)</sup>, and any beneficial actions of DHA have generally been attributed to a dampening effect on inflammatory mediators. Moreover, DHA could have useful effects by up-regulating the expression of genes involved in fatty acid oxidation while simultaneously down-regulating genes related to lipid synthesis<sup>10,11)</sup>.

The present study is an extension to our previous work and was designed to investigate the effects of DHA on NASH progression in our NASH rat model. We also examined whether changes in the ratio of n-6/n-3 polyunsaturated fatty acids in the liver is, associated with the development of NASH. Finally, we investigated whether DHA suppresses oxidative stress-induced damage of the liver including inflammation and fibrosis during NASH development.

## 2 MATERIALS AND METHODS

### 2.1 Materials

#### 2.1.1 Chemicals

DHA46-RD (46% purity) was kindly provided by Ikeda Tohka Industries Co (Fukuyama, Japan). DHA (46%) composed of myristic acid (14:0) 2.4%; palmitic acid (16:0) 11.8%; palmitoleic acid (16:1) 2.6%; stearic acid (18:0) 3.8%; oleic acid (18:1) 6.6%; linoleic acid (18:2) 0.8%;

$\alpha$ -linolenic acid (18:3) 0.3%; arachidonic acid (20:4) 2.5%; eicosapentaenoic acid, (20:5) 5.6%; docosapentaenoic acid (22:5) 1.4%; docosahexaenoic acid (22:6) 46.3% and others 15.9% (Table 1). All other reagents were obtained from Wako Pure Chemical Industries (Osaka, Japan). All chemicals used in this study were of the highest grade available.

### 2.2 Experimental animals

Male Wistar rats, six weeks of age and weighing 160-170 g, were purchased from Shimizu Experimental Animals (Shizuoka, Japan). The animals were housed at the Animal Research Center of Okayama University in a temperature-controlled room ( $22 \pm 1^\circ\text{C}$ ) with a relative humidity of  $50 \pm 10\%$  and a 12 h light/dark cycle (lights on at 8:00 a.m.). The Ethics Review Committee of Animal Experimentation of the Graduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama University approved the study protocol.

### 2.3 Plasma lipid peroxidation assay

The rats were divided at random into four groups of six rats each: Control, Soybean, DHA 100, and DHA 300. All rats were fed standard chow (MF diet) for 5 weeks. In addition, DHA was instilled orally at 100 or 300 mg/kg/day for 5 weeks in the DHA 100 and DHA 300 group, respectively. At the end of the 5-week treatment period, the rats were sacrificed by deep anesthesia and collected the blood samples. Diluted plasma was added to Luminol (130  $\mu\text{g/mL}$ ) and analyzed in an Auto Lumat device, Tristar LB941 (Berthold Technologies, Germany), at  $37^\circ\text{C}$  for 5 min, and then 0.33 mM tert-butyl hydroperoxide (*t*-BuOOH) was added, and then chemiluminescence (CL) intensity was detected for 120 min with incubating at  $37^\circ\text{C}$ . Each data denotes the

**Table 1** Fatty Acid Composition in MF Diet, CDHF Diet and DHA46-RD.

	MF (%)	CDHF (%)	DHA46-RD (%)
14:0	0.7	1.8	2.4
16:0	14.3	23.9	11.8
16:1	1.0	2.9	2.6
18:0	2.6	11.9	3.8
18:1n-9	25.5	43.0	6.6
18:2n-6	45.2	9.0	0.8
18:3n-3	3.3	0.5	0.3
20:4n-6	0	0.1	2.5
20:5n-3	1.0	0	5.6
22:5n-3	0.1	0	1.4
22:6n-3	0.7	0.3	46.3
others	5.6	6.6	15.9
Total	100.0	100.0	100.0

mean  $\pm$  SEM of the accumulated CL intensity for 120 min.

## 2.4 NASH model (PCT/JP 2007/52477) rat

Through the experiment period (10 weeks), rats were fed the same diets, either a MF diet (control group;  $n=6$ ) or a choline deficient high-fat (CDHF) diet (CDHF alone, NASH and NASH + DHA groups;  $n=6$ ). MF and CDHF diets were obtained from Oriental Yeast Co., Ltd. MF diet composed of casein (20%); corn starch (15%); corn oil (5%); sucrose (50%); mineral mix (3.5%); vitamin mix (1.0%); choline bitartrate (0.2%); DL-methionine (0.3%). CDHF composed of casein (8%); lard (38%); sucrose (48.4%); mineral mix (4.0%); vitamin mix (1.0%); choline bitartrate (0%); DL-methionine (0%). These fatty acids composition showed as **Table 1**.

Fatty liver rats were prepared by 4 weeks CDHF diets. The fatty liver rats were divided into 3 groups (6 rats/ each group) and were injected intraperitoneally for the subsequent 6 weeks, with physiological saline (CDHF group) or with sodium nitrite (50 mg/kg/day) (NASH group and the NASH + DHA group). For the NASH + DHA group, the oral DHA (1.0 g/kg/day) administration was performed concurrently during the period of nitrite injection. The control group was fed standard chow (MF) for the subsequent 10 weeks. At the end of the 10-week treatment period, the rats were fasted overnight and sacrificed by deep anesthesia with diethyl ether. Liver and blood samples were taken for biochemical and histological assessments of oxidative stress-related injury and the efficacy of DHA administration. The liver samples were fixed in 20% formalin for histological examination or frozen immediately to determine lipid composition and antioxidant activities.

## 2.5 Biochemical determinations of analyses liver damage

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities in plasma were measured using commercial enzyme assay kits (Wako)<sup>3, 4</sup>. Plasma alkaline phosphatase (ALP) levels and choline esterase (ChE) were determined using standard assays<sup>3, 4</sup>.

## 2.6 Plasma SOD-like activity

Superoxide dismutase (SOD)-like activity in plasma was measured using electron spin resonance (ESR) spectroscopy. Superoxide anion radicals were generated using a hypoxanthine-xanthine oxidase system. CYPMPPO 2-(5, 5-Dimethyl-2-oxo-2 $\lambda$  5-[1,3,2]dioxaphosphinan-2-yl)-2-methyl-3,4-dihydro-2H-pyrrole 1-oxide 1/2 was used as the spin-trapping reagent of superoxide anion radicals. The signal intensity was confirmed by the ratio of the height of the internal manganese standard signal and the fourth of eight peaks from the CYPMPPO-OOH spin adducts. The conditions used were as follows: magnetic field, 331.5 mT; power, 8 mW; modulation frequency, 9.41 GHz; modulation amplitude, 1  $\times$  0.1 mT; response time, 0.1 s; amplitude, 790;

sweep width, 10 mT; sweep time, 4 min; room temperature<sup>3, 4</sup>.

## 2.7 Histological examination

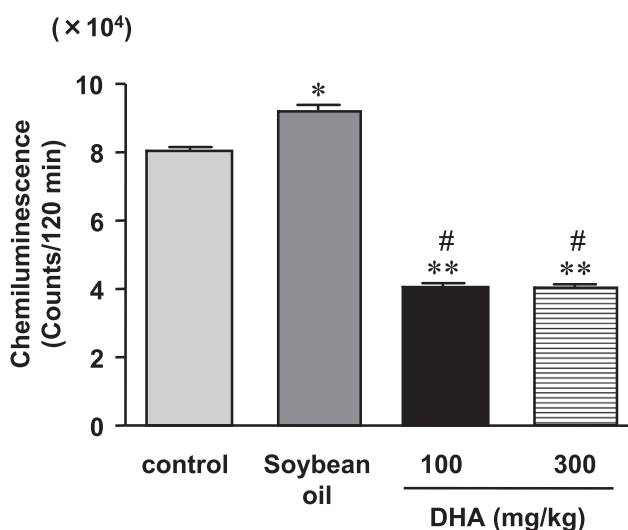
Paraffin-embedded tissues were sectioned at a thickness of 4  $\mu$ m and stained with Masson trichrome staining to visualize fibrosis. Tissue morphology was assessed histologically under light microscopy (Olympus, Tokyo, Japan)<sup>3, 4</sup>.

## 2.8 Analyses of fatty acid composition and total lipid content

Frozen liver tissue was homogenized using a polytron PT1200E (Kinematica, Switzerland), and then subjected to lipid extraction using the method of Folch *et al.*<sup>13</sup>. The samples were then dried by rotary evaporation and the liver tissue lipids were methyl-esterified for fatty acid analysis by gas chromatography<sup>14</sup>.

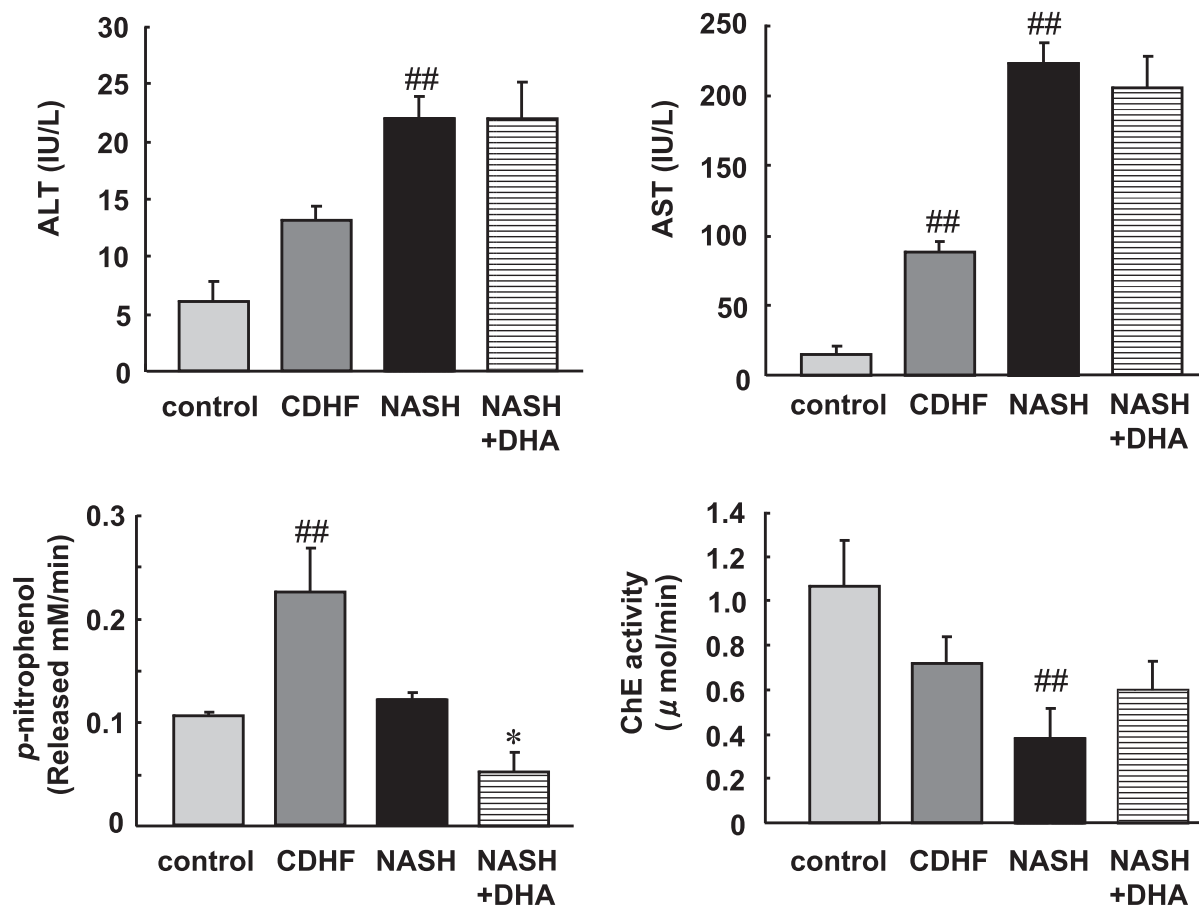
## 2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism (Version 4.03; Graph Pad Software, La Jolla, CA) on untransformed data. Data are expressed as mean  $\pm$  standard error of the mean (SEM). The groups were compared using analysis of variance with Dunnett's test as a post hoc test. Differences were considered significant when the probability value was less than 0.05.



**Fig. 1** Plasma Lipid Peroxide Levels in Rats Fed DHA or Soybean Oil.

Lipid peroxidation was estimated by *t*-BuOOH-initiated CL. Values represent the mean  $\pm$  SEM of 3 rats per group. \*\* $P<0.01$ , \* $P<0.05$  versus control, # $P<0.05$  versus Soybean oil.



**Fig. 2** Effects of DHA on Biochemical Markers in the Plasma of Control, CDHF, NASH and NASH+DHA. Values represent the mean±SEM of 6 rats per group. ## $P$ <0.01 versus control, \* $P$ <0.05 versus NASH. control: standard diet, CDHF: choline-deficient high-fat diet, NASH: CDHF+oxidative stress.

**Table 2** Effects of DHA on Body Weight and Liver Index.

	control	CDHF	NASH	NASH+DHA
Body weight (g)	333.2±6.7	206.2±6.2	211.6±10.1	204.5±10.7
Liver wet weight (g)	8.7±0.2	12.3±0.6	9.4±1.0*	13.9±1.8§
Liver index (%)	2.6±0.1	5.9±0.2	4.4±0.4**	6.7±0.7§

Values represent the mean±SEM of 6 rats per group. \* $p$  <0.05, \*\* $p$  <0.01, versus CDHF, § $p$  <0.05, versus NASH.

### 3 RESULTS

#### 3.1 Effect of DHA on plasma lipid peroxidation

The levels of plasma lipid peroxide in soybean oil-fed rats was significantly higher than in the control group, and DHA treatment significantly decreased these levels compared with the soybean oil group (Fig. 1).

#### 3.2 Effects of DHA on plasma biochemical parameters of liver damage

DHA administration significantly reduced ALP activities compared with NASH group rats (Fig. 2). However, DHA did not change the levels of typical markers for NASH (AST,

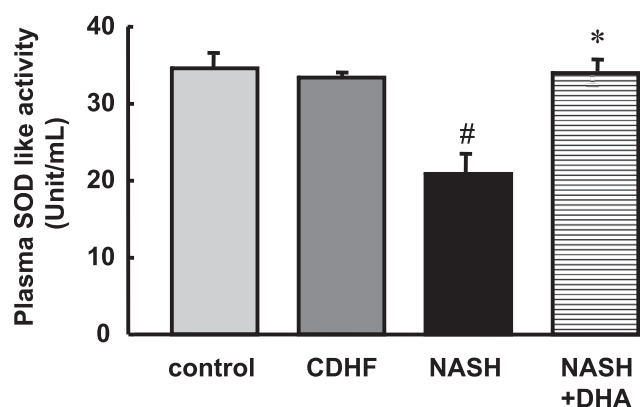
ALT, and ChE).

#### 3.3 Effects of DHA on the body weight and liver index

There were no differences in body weights among CDHF alone, NASH and NASH + DHA. In contrast, the body weights of rats fed standard chow were greater during the entire experimental period. Wet liver weights and liver indexes of NASH rats were lower than those of the NASH + DHA group and CDHF alone group (Table 2).

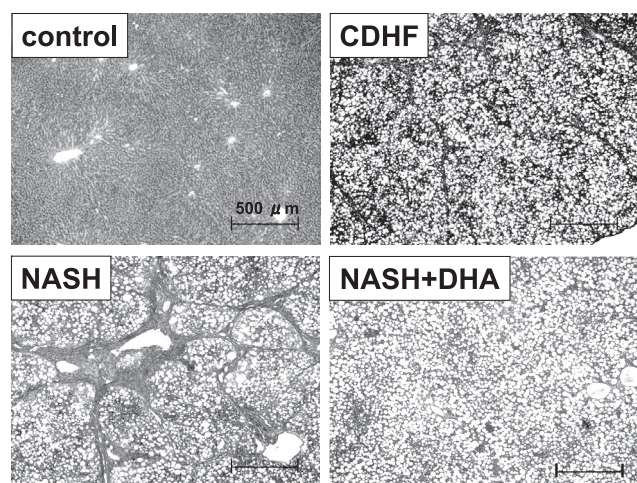
#### 3.4 Effects of DHA on plasma SOD-like activity

The levels of plasma SOD-like activity were significantly



**Fig. 3** Effects of DHA on Plasma SOD-like Activity in Control, CDHF, NASH and NASH+DHA Groups Using an ESR.

ESR spectra of the CYPMPPO-OOH spin-adducts obtained from hypoxanthine-xanthine oxidase systems in control, CDHF, NASH and NASH+DHA. Each value is the mean $\pm$ SEM of 6 rats per group. # $p$ <0.05, versus control. \* $p$ <0.05, versus NASH.



**Fig. 4** Histological Evaluation of Rat Liver.

Effects of DHA on liver fibrosis were examined by Masson trichrome staining in control, CDHF, NASH and NASH+DHA rat livers. Data show typical results. Scale bar=500  $\mu$ m.

lower in NASH rats. NASH rats supplemented with DHA at 1.0 g/kg showed a significant improvement and had high plasma SOD-like activity that was comparable to that found in NASH rats (Fig. 3).

### 3.5 Histological findings in liver samples

Typical liver specimens with Masson trichrome stain are shown in Fig. 4. No fibrosis was apparent in the livers of control group compared with those of the CDHF one. However, the NASH rats showed advanced liver steatosis, fibro-

sis, and necrosis. DHA administration clearly attenuated the liver fibrosis (Fig. 4).

### 3.6 Fatty acid composition in the liver

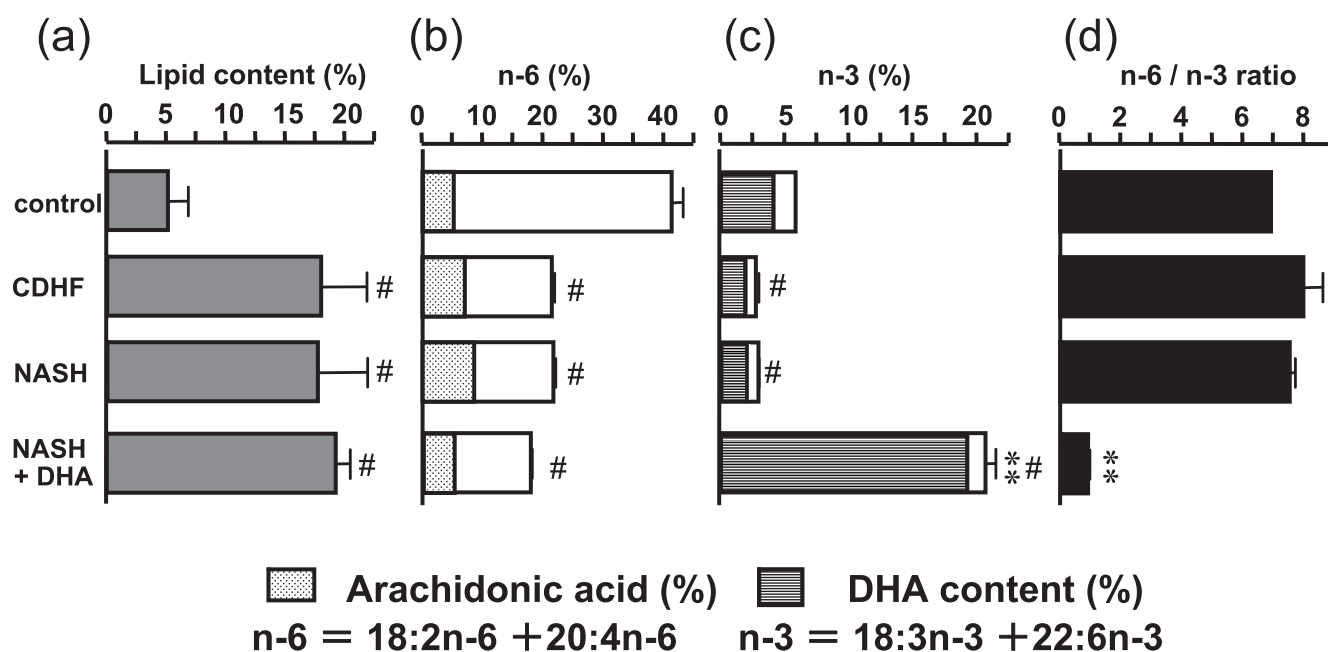
Lipids comprised 5% of the liver content in the control rats, and 16-18% in the other experimental groups. Therefore, it seemed that DHA treatment did not improve fatty liver, and as expected, the fatty acid profile of the liver reflected the dietary intake (Fig. 5). The C22:6n-3 fatty acid was decreased in CDHF and NASH groups compared with the control group, while rats fed chow supplemented with 1.0 g/kg DHA showed increased proportion of C22:6n-3. The C20:4n-6 fatty acids were increased in CDHF and NASH rat livers compared with the control group, and the C18:2n-6 fatty acids were decreased in all CDHF-fed rats compared with the untreated group. DHA treatment significantly decreased the n-6/n-3 ratio compared with other groups. There was no difference in other fatty acid profiles among CDHF, NASH, and DHA rats (Fig. 5).

## 4 DISCUSSION

The present study demonstrated that DHA administration attenuated NASH progression in an experimental NASH model rat, through reducing the content ratio of the n-6/n-3 polyunsaturated fatty acids in the liver. This finding is consistent with previous reports<sup>15)</sup>, and supports the notion that liver fatty acid composition is a key factor in NASH progression. To our knowledge, this is the first report of the preventive effect of DHA on NASH progression in an experimental rat model of the disease. We propose that changes to hepatic fatty acid composition play an important role in the mechanisms underlying NASH development.

In this study, we examined the effect of DHA on NASH progression. We showed that DHA administration increased plasma SOD like activity and suppressed the liver fibrosis, although plasma ALT and AST levels in NASH + DHA group were still high. These findings support that DHA develops an antioxidative role *in vivo*, although DHA is easily peroxidized<sup>16)</sup>. As we previously reported<sup>2, 4)</sup>, our model caused the increment of oxidative stress during NASH development. The increment of oxidative stress productions activates the hepatic stellate cell, resulting in fibrogenesis<sup>11, 18)</sup>. Therefore, we believe that the preventive effect of DHA could be mediated by the decrease of oxidative stress. However, we don't know why DHA administration couldn't influence of plasma AST and ALT levels.

One of the most important findings of the present study was that the DHA intake lowered n-6/n-3 polyunsaturated fatty acids ratio, and thereby delayed NASH progression. Recent studies reported significant accumulation of triacylglycerols in the livers of NAFLD patients compared to



**Fig. 5** Effects of DHA on Fatty Acid Composition in the Liver of Control, CDHF, NASH and NASH+DHA.

Values represent the mean±SEM of 6 rats per group. <sup>#</sup>*P*<0.05 versus control, <sup>\*\*</sup>*P*<0.01 versus NASH.

healthy controls, as well as decreased long-chain polyunsaturated fatty acids, particularly of the n-3 series, in both whole liver extracts and hepatic triacylglycerol or phospholipid fractions<sup>19</sup>. NASH and NAFLD patients generally consume more saturated fat and carbohydrates, and less unsaturated fats than healthy weight-matched controls. It is therefore reasonable to speculate that the amount of saturated fat in the liver of NAFLD patients who progress to NASH would be increased<sup>20, 21</sup>. Thus, it is important to examine the relationship between intrahepatic fatty acid composition and liver damage in NASH. Here, we found an increased n-6/n-3 polyunsaturated fatty acid ratio in the liver of NASH rats compared with the control. In particular, arachidonic acid, a C20:4;n-6 fatty acid, was increased in CDHF and NASH rats compared with control rats, but C18:2, an n-6 fatty acid, was unchanged. On the other hand, DHA, a C22:6;n-3 fatty acid, was decreased in CDHF and NASH rats compared with the control. These results strongly implicate the decrease in n-3 PUFAs in NASH progression. It is possible that the decrease in n-6/n-3 ratios regulate prostaglandin metabolism related to the inflammatory response by preventing the arachidonic acid cascade. Therefore, depletion of DHA in NASH liver might facilitate the prevalence of fatty acids and triacylglycerol synthesis over fatty acid oxidation, leading to inflammation induced by oxidative stress. This finding is in agreement with previous reports already discussed<sup>5, 15</sup>, suggesting that DHA administration could suppress liver fibrosis during NASH development by changing the fatty acid composition and decreasing oxidative stress in the liver.

Recently, n-3 PUFAs were implicated in the conversion to a novel series of lipid mediators designated resolvins and protectins, which mediate the protective and beneficial effects of n-3 PUFA. Indeed, these n-3 PUFA-derived lipid mediators showed potent protective actions in an experimental brain ischemic reperfusion model and in obesity-induced insulin resistance and steatosis<sup>22-26</sup>. Of particular interest is resolvin E1, a representative member of these novel lipid autacoids. Resolvin E1 is the most effective drug candidate of the growing family of endogenous resolvins and the compound with the most developed biology. González-Pérez *et al.*<sup>26</sup> demonstrated that n-3 PUFAs and their bioactive derivative lipid mediators exert protective actions in the liver by preventing necroinflammatory injury. Thus, the suppressive effects of DHA on NASH development observed in the present study might involve these lipid mediators. However, further studies are warranted to assess the relationship between intrahepatic fatty acid composition and liver damage in NASH development, and ultimately to determine the mechanisms by which the antifibrotic activity of DHA improves NASH.

DHA46-RD is established safety and efficacy from a nutritional and preventive medicine viewpoint. In the present study, the dose of DHA was up to 100-fold higher than the DHA doses found to lower triacylglycerol levels in humans. Ryan *et al.* mentioned that doses of investigational product administered to animals are much higher than those given to human. Higher doses are needed to account for increased metabolic rates in rodents<sup>27</sup>. Therefore, we believe that the toxicity of DHA used in this study might be negli-

gible. However, further research is needed to demonstrate efficacy and safety of DHA46-RD.

## 5 CONCLUSION

In the present study, the fish oil-derived DHA ameliorated the progression of NASH in a rat model of the disease by lowering oxidative stress and the n-6/n-3 polyunsaturated fatty acid ratio in the liver. These findings suggest that a decrease in n-3 polyunsaturated fatty acids contributes to the progression of NASH via the associated oxidative stress-induced damage and inflammation. Further studies are needed to establish the therapeutic value of DHA-containing food in prevention or amelioration of NASH.

## ACKNOWLEDGMENT

We thank Ikeda Tohka Industries Co., Ltd. for providing the DHA. We also thank Ms. Keiko Ihara, Mr. Shinki Murakami and Ms. Ayaka Sato for the excellent technical assistance. This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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