Original Article

Effect of tocotrienol on the primary progression of nonalcoholic steatohepatitis in a mouse model

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Tocotrienol (T3), a vitamin E (Vit E) isoform, is known to have both biological and antioxidant effects. Although alpha-tocopherol (α-Toc), another isoform of Vit E is suggested to be a useful treatment against nonalcoholic steatohepatitis (NASH), the effect of T3 on NASH is unclear. This study aimed to comparatively evaluate the effects of T3 and α-Toc on NASH in the early stage of NASH progression, using a recently established NASH mouse model induced by a choline-deficient L-amino acid-defined high-fat diet (CDAHFD). Six-week-old male mice were divided into four groups (*n* = 6 per group) and fed the CDAHFD for 1 week. The first group was given no other treatment (Pre). The other three groups continued the CDAHFD plus daily oral administration of Vit E-free corn oil (Control), corn oil containing α-Toc, or corn oil containing T3 for additional 2 weeks. Neither Vit E treatment changed the histologic features of NASH, but T3 significantly reduced the mRNA expression of several genes related to inflammation and fibrosis and α-Toc did not. These results suggested that oral T3 treatment was more effective than α-Toc at suppressing hepatic inflammation and fibrosis in the early stage of NASH progression in CDAHFD model mice.

***Key Words*: vitamin E, tocotrienol, nonalcoholic steatohepatitis (NASH), inflammation, choline-deficient L-amino acid-defined high-fat diet**

Nonalcoholic fatty liver disease (NAFLD), characterized as the clinical state of triglyceride accumulation or steatosis, is a chronic liver disease associated with obesity, insulin resistance, and diabetes. NAFLD consists of two clinical entities, simple benign steatosis and nonalcoholic steatohepatitis (NASH). NASH can progress to cirrhosis or hepatocellular carcinoma.[1] As NAFLD/NASH has been increasing and becoming a global issue, it is important to prevent the progression of simple fatty liver to NASH.

Other than changing lifestyle, there is no therapeutic treatment for NASH at present because long-term medication has possible adverse effects. The results of several clinical trials to treat NAFLD/NASH suggest that vitamin E (Vit E) is useful.[2] The PIVENS trial was a large-scale analysis of the effects of Vit E and also compared those effects to those of pioglitazone. The trial showed that Vit E in the form of α-tocopherol (α-Toc) administered at a dose of 800 IU/day for 96 weeks to adults with NASH was associated with a decrease in serum aminotransferases and histological improvement in steatosis, inflammation, and ballooning, as well as with the resolution of steatohepatitis.[3] Although two different meta-analyses produced conflicting results,[4,5] Vit E is recommended by the guidelines of NICE (National Institute for Heat and Care Excellence) and AASLD (American Association for the Study of Liver Diseases).[6]

Vit E is composed of a chromanol ring and an isoprenoid chain with 16 carbon atoms. Those structures with saturated side chains are referred to as tocopherols, and those with unsaturated side chains are called tocotrienols. Also, depending on the number and places of methyl groups on the chromanol ring, the Vit E isoforms are separated into four types, α, β, γ, and δ, resulting in a total of eight isoforms. Even though all of these Vit Es have similar antioxidant functions, only α-Toc is believed to have a biological function as Vit E *in vivo* because of its discrimination by the hepatic α-tocopherol transfer protein (α-TTP) in liver.[7] On the other hand, tocotrienol (T3) accumulates in adipose tissue and skin because of the lipophilicity of its unsaturated side chain,[8] and it is also known for effects such as anti-inflammation,[9] anti-cancer,[10] and inhibition of lipid synthesis,[11] in addition to antioxidant effects. Therefore, we speculate that T3 might act in fatty liver and prevent the progression of NASH.

One of the most common animal models used in NASH research is feeding a diet deficient in both methionine and choline (MCD diet), leading to fatty liver by the inhibition of very-low-density lipoprotein (VLDL) secretion. However, this model results in severe body weight loss and liver atrophy, which are not characteristics of human NASH. In order to improve this problem, the choline-deficient L-amino acid-defined high-fat diet (CDAHFD) was established.[12] The CDAHFD increased the plasma levels of alanine aminotransferase in mice after 7 days, and by 6 weeks the mice had developed enlarged fatty liver with fibrosis, suggesting that CDAHFD feeding is a useful model for the assessment of NASH, especially rapidly progressive liver fibrosis.

Several studies using rat models[13,14] suggested that Vit E is effective, but a comparison of various animal models caused by different disease inductions is needed, because NASH presents a complex pathology. Therefore, our study aimed to comparatively evaluate the effects of T3 and α-Toc in the early stage of NASH promotion, using the improved NASH model mouse induced by CDAHFD.

Materials and Methods

Materials. C57BL/6J mice were obtained from Clea Japan, Inc. (Tokyo, Japan). A CDAHFD with 0.1% methionine (A06071302; Research Diets, New Brunswick, NJ) was purchased, and its composition is shown in Table 1. α-Toc (above 99.8% purity) and a T3 mixture (32% α-T3, 5% β-T3, 48% γ-T3, and 15% δ-T) were provided by MITSUBIHSHI CHEMICAL FOODS Co., Ltd. (Tokyo, Japan). Vit E-free stripped corn oil was kindly donated by Tama Biochemical (Tokyo, Japan).

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Table 1. Composition of the CDAHFD

Ingredient	(g)		(g)
L-Cystine	4.2	Casein	0
L-Isoleucine	7.6	Corn starch	0
L-Leucine	15.8	Maltodextrin	130.1
L-Methionine	13.2	Sucrose	68.8
L-Lysine	0.8	Cellulose	50.0
L-Phenylalanine	8.4	Soybean oil	25.0
L-Threonine	7.2	Lard	245.0
L-Tryptophan	2.1	Mineral mix S10026	10.0
L-Valine	9.3	Dicalcium phosphate	13.0
L-Histidine	4.6	Calcium carbonate	5.5
L-Alanine	5.1	Potassium citrate	16.5
L-Arginine	6.0	Sodium Bicarbonate	7.5
L-Aspartic acid	12.1	Vitamin mix V10001	10.0
L-Glutamic acid	38.2	Choline bitartrate	0
Glycine	3.0		
L-Proline	17.8		
L-Serine	10.0		
L-Tyrosine	9.2	Total	756.05

CDAHFD, Choline-deficient L-amino acid-defined high-fat diet with 0.1% methionine.

Animal experimental design. All the experimental procedures were approved by The Animal Ethics Committee of Ochanomizu University. Five-week-old male C57BL/6J mice were maintained under standard laboratory conditions by feeding normal chow (CE2; CLEA Japan, Tokyo). After 7 days of acclimation, the mice were randomly divided into four groups ($n = 6$ per group) and were then fed the CDAHFD. The mean body weights were similar between groups at week 0 of the experiment. One of the groups finished this experiment in one week (Pre), and the other three groups continued the CDAHFD with an oral Vit E administration every day for another two weeks. Mice in the control group were given 0.1 ml of Vit E-free corn oil. Those in the α-Toc and T3 groups were administered Vit E-free corn oil containing α-Toc (4 mg/0.1 ml oil) and T3 (4 mg/0.1 ml oil), respectively.

At the end of the 3-week experimental period, the mice were killed after 12 h of fasting, and the blood, liver, and white adipose tissues of the epididymis, kidney, and mesenterium were collected. Serum was separated by centrifugation and stored at −80°C. Collected tissues were weighed, frozen in liquid nitrogen, and stored at −80°C. Part of the liver was embedded in paraffin.

Serum parameter measurements. Concentrations of serum triglyceride (TG) and total cholesterol (TC) and activities of alanine transaminase (ALT) and aspartate aminotransferase (AST) were measured using a Fuji Dry-chem 4000V chemistry analyzer (Fujifilm Corp., Tokyo).

Liver histological evaluation. Liver tissue was dipped in 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS) overnight and fixed. Liver tissue sections embedded in paraffin were subjected to three types of staining. NASH pathological findings are characterized by infiltration of inflammatory cells, ballooning of hepatocytes, Mallory bodies, and fibrosis around hepatocytes in addition to large droplet fat deposition on hepatocytes. Hematoxylin and eosin (H&E) staining was performed to observe liver conditions. Azan staining was performed to clarify the progression of liver fibrosis by staining collagen fibers accumulated by liver fibrosis. Sirius red staining was performed to quantify collagen fibers to grasp the state of liver fibrosis. After staining, liver tissue sections were observed under a BZ-X700 fluorescence microscope (Keyence Corp., Osaka, Japan).

RNA isolation, reverse transcription, and real-time PCR. Real-time PCR was performed to determine the mRNA expression of various genes in liver. First, RNA was isolated from liver tissue by ISOGEN (Nippon Gene, Tokyo. Japan) and absorbance was measured using a Biospec Nano spectrophotometer (Shimadzu Corp., Kyoto, Japan). RNA was reverse transcribed using ReverTra Ace qPCR RT Master Mix (FSQ-201; Toyobo, Osaka, Japan) and cDNA was synthesized. Real-time PCR was performed by Thunderbird SYBR qPCR Mix (QPS-201; Toyobo) according to the Thunderbird SYBR qPCR/RT Set protocol and analyzed using the Step One Plus™ Real-Time PCR System (Thermo Fisher Scientific K.K., Tokyo). Each gene was corrected using glyceraldehyde-3-phosphate dehydrogenase (Gapdh) as an endogenous control. The primer sequences used are listed in Table 2.

Measurement of Vit E concentrations in the liver. Vit E concentrations in liver were measured using high-performance liquid chromatography (HPLC) (20-AD; Shimadzu Corp.) and a fluorescence detector (RF-10 AX; Shimadzu Corp.) according to the method previously described.[15]

Statistics. Statistical analysis was performed by one-way analysis of variance and multiple comparison test by the Tukey-Kramer method. Statistical significance was defined as $p<0.05$, and values are shown as means ± SE. All statistical analyses were performed using Ekuseru-Toukei statistical software (Social Survey Research Information Co., Tokyo).

Results

Body weight change during the experimental period. The body weight of mice in all groups did not change throughout the experiment (Table 3), suggesting that the CDAHFD had an advantage over the MCD diet for the NASH model mice. Neither α-Toc nor T3 treatment led to significant changes in body weight or tissue weight (Table 3). When mice were fed the CDAHFD for 6 weeks, the liver accumulated fat gradually for 1 week, 3 weeks,

Table 2. Primer sequences used in this study

Gene	Forward	Reverse
Mouse F4/80	5'-TGTGTCGTGCTGTTCAGAACC-3'	5'-AGGAATCCCGCAATGATGG-3'
Mouse *Tnfα*	5'-CATCTTCTCAAAATTCGAGTGACAA-3'	5'-TGGGAGTAGACAAGGTACAACCC-3'
Mouse *Col1a1*	5'-TTCCCTGGACCTAAGGGTACC-3'	5'-CTGAGCTCCAGCTTCTCC-3'
Mouse *Col4a1*	5'-CTGGAGAAAAGGGCCAGAT-3'	5'-TCCTTAACTTGTGCCTGTCCA-3'
Mouse α-SMA	5'-ATCGTCCACCGCAAATGC-3'	5'-AAGGAACTGGAGGCGCTG-3'
Mouse *Mmp9*	5'-CCATGCACTGGGCTTAGATCA-3'	5'-GGCCTTGGGTCAGGCTTAGA-3'
Mouse *Mmp13*	5'-CCAGAACTTCCCAACCATGT-3'	5'-GTCTTCCCCGTGTTCTCAAA-3'
Mouse *Gapdh*	5'-AACTTTGGCATTGTGGAAGG-3'	5'-CACATTGGGGGTAGGAACAC-3'

Table 3. Changes in body weight during experimental period in mice fed CDAHFD (g)

	0 week	1 week	2 weeks	3 weeks
Pre	20.3 ± 0.31	19.7 ± 0.33	—	—
Control	20.2 ± 0.24	19.0 ± 0.14	18.8 ± 0.24	18.8 ± 0.32
α-Toc	20.2 ± 0.27	19.8 ± 0.20	19.3 ± 0.26	20.0 ± 0.26
T3	20.3 ± 0.44	19.9 ± 0.39	19.4 ± 0.37	20.1 ± 0.22

All the groups of mice were fed by CDAHFD and further administered orally Vit E free oil (Control), α-Toc and T3. The results are expressed as means ± SEM (n = 6).

Table 4. Tissue weight and serum parameter in mice fed CDAHFD

	Pre	Control	α-Toc	T3
Tissue weight				
Liver (g)	1.09 ± 0.031	1.10 ± 0.055	1.28 ± 0.046	1.33 ± 0.020
Liver/body weight (g/g)	0.055 ± 0.0007	0.058 ± 0.0022	0.064 ± 0.0019	0.066 ± 0.0009
Adipose tissue, epididymal (g)	0.19 ± 0.023	0.20 ± 0.040	0.25 ± 0.021	0.18 ± 0.018
Adipose tissue, perirenal (g)	0.03 ± 0.008	0.04 ± 0.008	0.05 ± 0.006	0.04 ± 0.009
Adipose tissue, mesenteric (g)	0.04 ± 0.014	0.06 ± 0.008	0.08 ± 0.007	0.07 ± 0.016
Serum parameter				
TG (mg/dl)	58.5 ± 2.20^{ab}	49.3 ± 3.43^{a}	60.7 ± 3.06^{ab}	64.0 ± 3.30^{b}
TC (mg/dl)	66.5 ± 1.77	66.0 ± 2.46	67.2 ± 3.02	70.7 ± 5.22
ALT (IU/L)	217 ± 15.9^{a}	291 ± 26.0^{a}	408 ± 40.5^{b}	263 ± 22.1^{a}
AST (IU/L)	178 ± 11.7^{a}	241 ± 15.7^{ab}	267 ± 27.7^{b}	187 ± 6.33^{a}

The results are expressed as means ± SEM (n = 6). Means without a common letter are significantly different, p<0.05.

and 6 weeks (Supplemental Fig. 1*). Since it was considered that large lipid droplets were accumulated and changes in the initial state of NASH were observed after 3 weeks of CDAHFD feeding, we decided to observe changes after 3 weeks of feeding.

Tissue weight and serum parameters. Liver weight, the ratio of liver weight to body weight, and white adipose tissue weight were similar among all groups (Table 4). Concentrations of TG and TC in serum did not differ across groups. Levels of ALT and AST worsened with NASH progression, but not so much in the T3 group (Table 4).

Pathological features of NASH in the experimental diets. In our previous study, the CDAHFD led to fat accumulation after one week, and large fat droplets gradually increased from 3 weeks to 6 weeks (Supplemental Fig. 1*). Fibrosis was also observed at 6 weeks of CDAHFD feeding (Supplemental Fig. 1D*). In the present study, to confirm the effects of Vit E on early and ongoing NASH, we set the experimental period to 3 weeks and observed greater increases in liver fat accumulation in mice fed the CHDHFD for 3 weeks than in mice fed CDAHFD for 1 week (Fig. 1). There was no significant difference in the triglyceride concentrations in the liver (data not shown) during the 3-week experimental period.

Gene expression in liver related to inflammation and fibrosis. We evaluated the mRNA expression of inflammation-related genes to confirm the inflammatory state of the liver (Fig. 2A). The levels of F4/80, an inflammatory cell activation marker, were significantly increased after the 3-week CDA HFD treatment; however, both Vit E isoforms, but especially the T3 treatment, suppressed the induction of F4/80 expression. The effects of α-Toc and T3 on tumor necrosis factor a (TNFα) expression showed tendencies similar to those of F4/80. Among fibrogenesis-related genes, Collagen type I (Col1a1) tended toward a low value in the T3 group. In addition, Collagen type IV (Col4a1) showed a significantly low value in the T3 group (Fig. 2B). Col1a1 is a fibrillar collagen and is the most abundant collagen in vertebrates. On the other hand, collagen type IV is a membrane-type collagen and present in the basement membrane. Normally, there is no basilar membrane in sinusoids of the liver, but in liver disease, proliferation of the basement membrane around the sinusoid is observed. However, the levels of α-smooth muscle actin (α-SMA), an activator stellate cell marker, did not differ among the groups. α-SMA also showed no difference in immunostaining (data not shown). Regarding fibrinolysis, the level of matrix metalloproteinase 9 (MMP9) was significantly lower in the T3 group and MMP13 was also lower in the T3 group (Fig. 2C). MMP9 breaks down collagen types III, IV, and V, and MMP13 breaks down collagen types I, II, and III. The low expression of these two fibrinolytic marker genes, MMP9 and MMP13, may have been due to the fact that fibrosis in the liver was not progressing and therefore the necessity of decomposing the fiber was lower in this group than in the others.

Histological features of fibrosis in the liver. Because we observed differences in fibrosis-related genes, we performed two types of staining to confirm the fibrosis state of the liver. Azan staining and Sirius red staining were performed for histologic evaluation (Fig. 3A and B). Both stainings revealed that the fibrotic area was larger in the groups fed the CDAHFD for 3 weeks than in the Pre group. The result of Sirius red staining revealed that collagen fibers were more frequently found in the interstitial area of the Control group whereas in the groups administered α-Toc or T3, fewer dense collagen fibers tended to be found.

Orally administered Vit E accumulated in liver tissues. We measured the amounts of Vit E in the liver (Table 5). α-Toc was detected in all groups because the CDAHFD included α-Toc. The α-Toc group had more than three times the amount of α-Toc compared to the other groups. Tocotrienol was detected in only the T3 group. The α-T3 concentration was highest even though the administered T3 contained high γ-T3.

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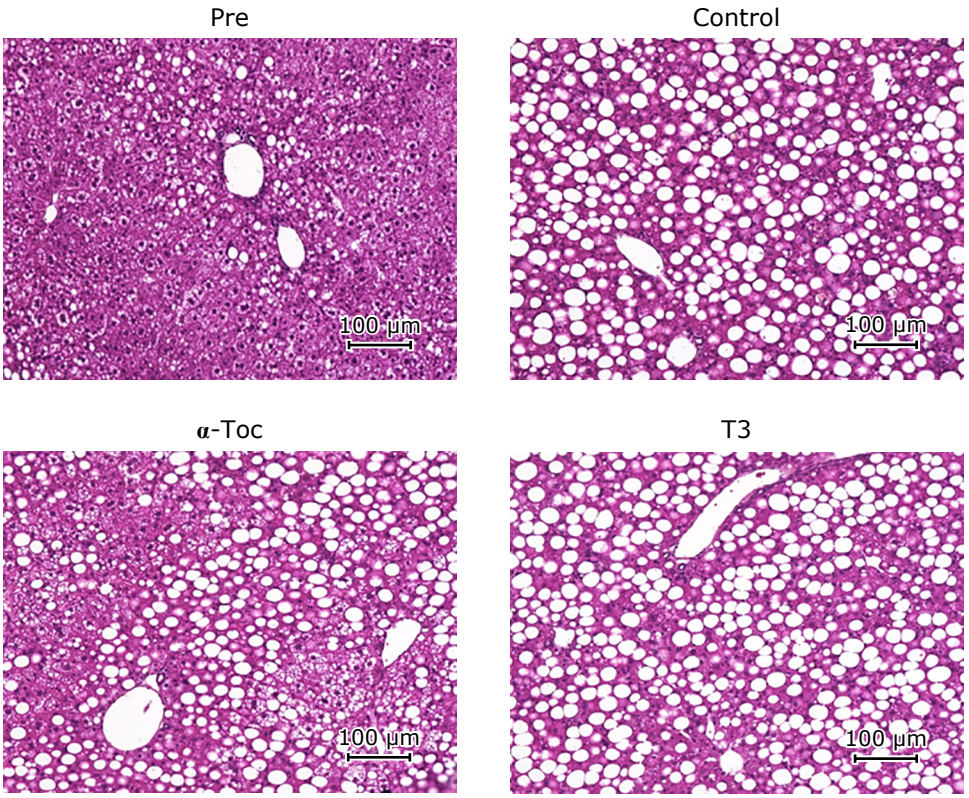

Fig. 1. Hepatic fat accumulation and inflammation in mice fed CDAHFD. Histopathology of mouse liver stained with hematoxylin-eosin. The liver of mice fed CDAHFD for 1 week as pretreatment (Pre) and fed 2 more weeks with no treatment (Control), oral administration of α-Toc (α-Toc), and tocotrienol (T3).

Discussion

There are many diet-induced NAFLD/NASH models. For example, a high-fat, high-carbohydrate diet and a high-fat diet plus water containing fructose and glucose induce NAFLD[16] but do not induce NASH, especially hepatic fibrosis. The methionine- and choline-deficient diet (MCD) is the most widely used diet that induces histopathological features that meet NASH diagnostic criteria such as steatosis, lobular inflammation, hepatocyte ballooning, and stromal fibrogenesis.[17,18] The MCD diet induces hepatic fat accumulation and inflammation, because methionine and choline are essential for VLDL secretion from the liver. Although MCD surely causes fat accumulation and inflammation in the liver, it has a large effect on the whole body, especially remarkable body weight loss. Therefore, MCD dose not reproduced a NASH state accurately and is difficult to use in long-term experiments. The CDAHFD,[12] which contains a small amount of methionine as a precursor to choline biosynthesis, causes VLDL secretion to an extent that does not cause weight loss. We have also confirmed the CDAHFD can reproduce all of the NASH-specific pathological features described above while maintaining body weight and the general condition (Supplemental Fig. 1*). Our results also showed no significant change in body weight (Table 2), liver weight, or white adipose tissue weight (Table 3). However, fat accumulation in the histological evaluation and inflammatory gene expression also tended to be higher in the control group than the in the Pre group (Fig. 1 and 2A). Thus, it is considered that the characteristics of CDAHFD are shown as they are. Although AST and ALT did not differ significantly between the Control and T3 groups, those in the T3 group had relatively low values. AST and ALT showed significantly higher values in the α-Toc group than in the other groups. In a previous report ALT and AST activities increased in NASH, but there was no correlation with the progression degree of NASH in either the CDAHFD model or in human NAFLD patients.[19]

Hepatic fat accumulation did not differ significantly among the groups in morphological observation or in the biochemical measurements of TG contents. Namely, Vit E administration did not affect hepatic fat accumulation during NASH progression in this study. This differs from the report by Phung *et al.*,[20] in which Vit E supplementation reduced steatosis in mice fed MCD. However, since the present study focused on NASH formation by CDAHFD feeding for 3 weeks, it looked only at the early stage of NASH. Since fat accumulation further progresses by CDAHFD feeding for 6 weeks (Supplemental Fig. 1*), it is possible that Vit E has an inhibitory effect on fat accumulation when the CDAHFD feeding period is further extended beyond 3 weeks. The gene expression of F4/80 as a lobular inflammation marker, which shows macrophage activity, was increased in the Control group with the progress of NASH. The T3 group significantly reduced F4/80 gene expression compared to the Control group and tended to also be lower than in the Pre group. In addition to its antioxidative function, the Vit E family can reduce inflammation, haptic stellate cell activation, fibrosis, and can decrease the NAFLD activation score in NAFLD.[21–23] Especially, T3 homologs have been reported to have anti-inflammatory effects.[9] Our results also indicated that oral administration of T3 during NASH progression suppressed hepatic inflammation.

In the T3 group, no change was seen in the gene expression of αSMA, which indicates the activation of hepatic stellate

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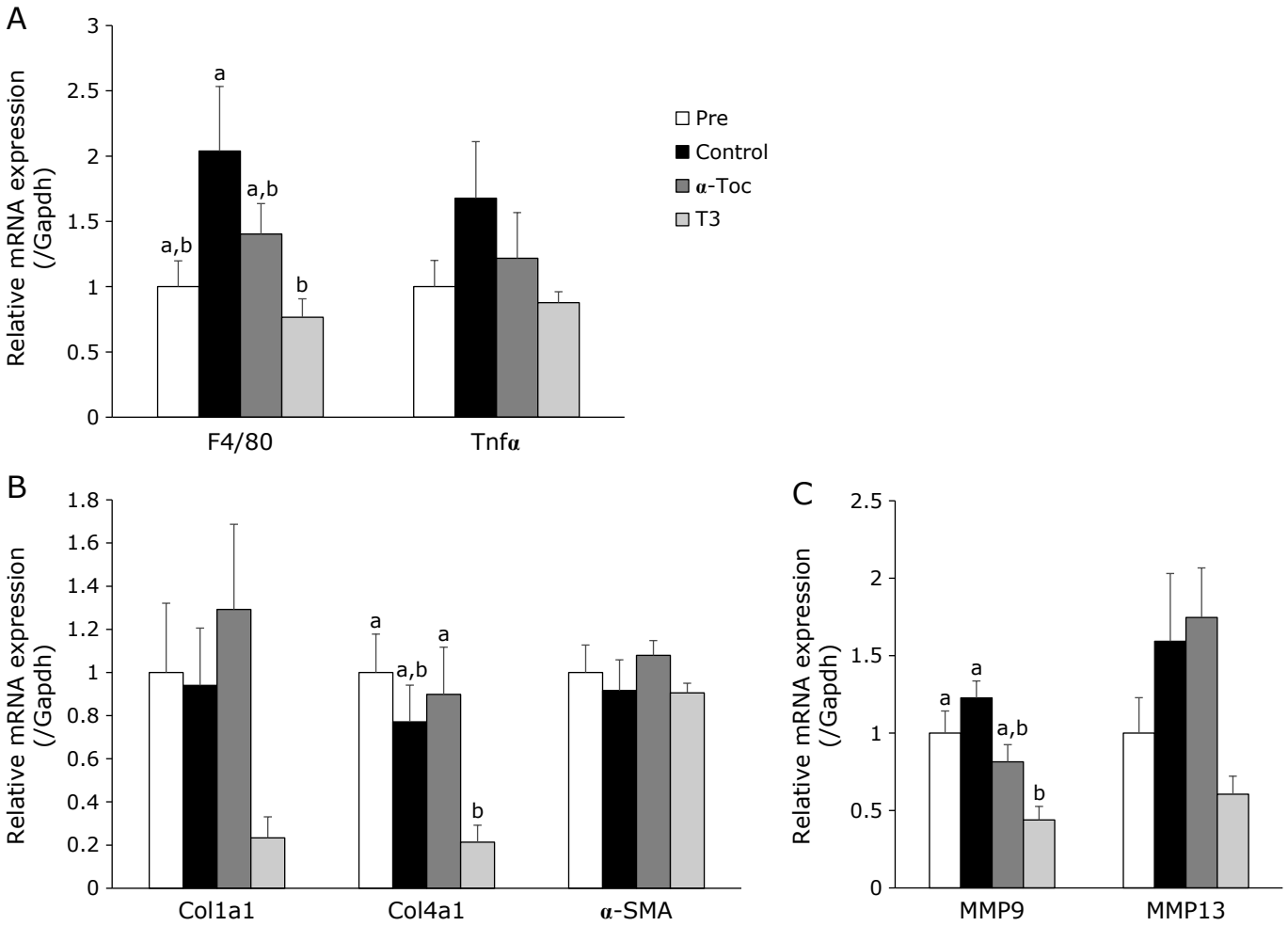

Fig. 2. Hepatic gene expression associated with inflammation in mice fed CDAHFD. Gene expression associated with inflammation (A), fibrosis (B), and fibrolytic activity (C). The results are expressed as means ± SEM (n = 6). Means without a common letter are significantly different, p<0.05.

cells. However, the Col1a1 and Col4a1 genes, which are the main genes involved in fibrogenesis, as well as MMP9 and 13, showed significantly lower values in the T3 group than in the Control group. MMP9 and MMP13 are expressed in liver with enhanced fibrosis and in atherosclerosis and contribute to tissue repair.[24–26] The low MMP expression in the T3 group was attributed to the significant suppression of inflammation and fibrosis. Morphometric analysis of collagen fiber expression level by Sirius red staining showed no significant difference among the groups (data not shown). However, although the distribution of collagen fibers was similar among the groups around the central vein, distribution to the stroma around the sinusoid was frequently observed in the Control group. During NASH progression, fibrosis is first observed in the perisinusoidal stroma in Zone 3 (perivenular area), after which fibrosis of the periportal area progresses to form bridging.[27] Stromal fibrosis, which indicates the progression of NASH, was observed in the Control group, and was considered to be suppressed in the T3 group. This suggested that T3 may suppress the progression of fibrosis during the early NASH formation process.

Previous studies using rat model also found that Vit E did not show histological improvement of hepatic fibrosis. Miyazaki *et al.*[13] fed Wistar rats an MCD diet containing α-Toc (500 mg/kg) for 4 weeks and reported that α-Toc did not change the mRNA expression levels of genes related to fibrosis, such as transforming growth factor β (TGFβ) and tissue inhibitor of metalloproteinase 1 (TIMP1), despite the reduction of hepatic lipid peroxidation. On the other hand, Muto *et al.*[28] showled that γ-T3 reduced TG levels in primary rat hepatocytes and rats fed a high-fat diet. Yachi *et al.*[14] found that T3 reduced hepatic TG levels and that gene expression of inflammation markers in a rat steatohepatitis model induced inflammation by injection of *N*-acetylgalactosamine and TNFα. Although the animal models were different, our study agreed with these results and suggested that T3 might be beneficial for inhibiting inflammation and fibrosis in the early stage of NASH progression. Kim *et al.*[21] suggested that the inhibition of fibrosis by γ-T3 might be mediated by endoplasmic reticulum (ER) stress on the basis of results using C/EBP homologous protein (CHOP)-deficient mice fed MCD to induce NASH. While they focused on γ-T3, we also need to mention α-T3 in our study.

We measured Vit E amounts in the liver to ascertain whether orally administered Vit E actually accumulated in the liver. The CDAHFD-derived α-Toc showed accumulation of 11 nmol/g in one week (Pre) and 21 nmol/g in 3 weeks (Control). α-Toc is known to accumulate in the liver without degradation by binding to α-TTPs.[29] Two weeks of oral administration resulted in the accumulation of 479 nmol/g of α-Toc in the α-Toc group, approximately 20 times that in the Control group. It has been said that T3 has a low affinity for α-TTP and is unlikely to accumulate in

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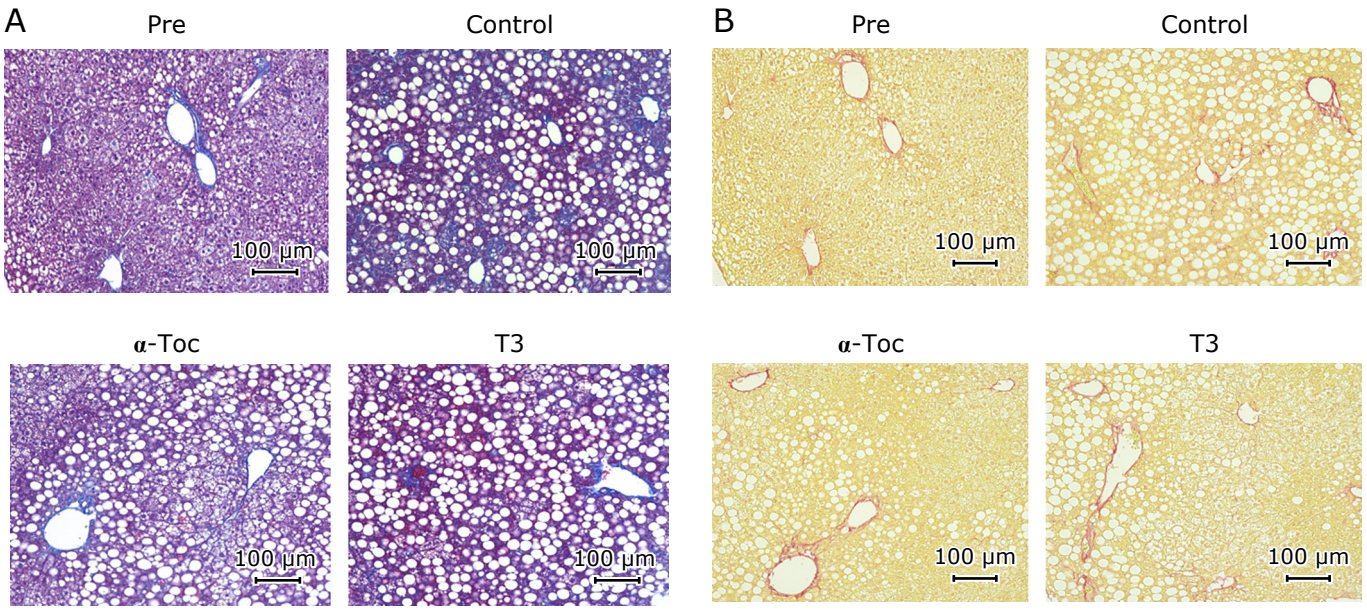

Fig. 3. Pathological feature associated with fibrosis in mice fed CDAHFD. Histopathology of mouse liver stained with Azan (A) and Sirius red (B). The liver of mice fed CDAHFD for 1 week as pretreatment (Pre) and fed 2 more weeks with no treatment (Control), oral administration of α-Toc (α-Toc), and tocotrienol (T3).

Table 5. Vitamin E concentrations in the liver after oral administration (nmol/g)

	α-Toc	α-T3	β-T3	γ-T3
Pre	11.1 ± 2.4[a]	ND	ND	ND
Control	20.6 ± 1.3[a]	ND	ND	ND
α-Toc	479 ± 47[b]	ND	ND	ND
T3	14.5 ± 0.8[a]	70.6 ± 8.2	1.89 ± 0.1	8.54 ± 0.9

Concentrations of each Vit E isoforms were measured by HPLC. The results are expressed as means ± SEM (n = 6). Means without a common letter are significantly different, p<0.05.

the liver. Vit E is normally metabolized nonspecifically in the liver.[30] α-TTP is considered to protect α-Toc from being metabolized in the liver by binding to α-Toc with a high affinity. Therefore, α-Toc is likely to accumulate in the liver, and other homologues are metabolized and disappear in the liver. In the present study, 71 nmol/g of α-T3 was detected in the T3 group. This was about 1.8 times the α-T3 levels accumulated in the liver at 8 h after a single administration of T3 (data not shown). Therefore, it is considered that α-T3 gradually accumulated in the liver after administration for 2 weeks. Although a T3 mix was used in this study, the amount of accumulated α-T3 was greater than the accumulated amounts of any of the other homologues, suggesting that α-T3 has a high affinity for α-TTP among T3 homologues. On the other hand, β- and γ-T3s also remained without complete degradation in the liver. Therefore, the NASH model used in this study was beneficial for evaluating the histochemical effect of T3 *in vivo*. From these considerations, we can infer that the accumulation of T3s in the liver in the T3 group suppressed the onset of inflammation and fibrosis in the early stage of NASH. Unfortunately, in the present study, T3 was administered as a mixture, so it is not possible to determine which T3 homologue is responsible for this suppression.

In a recent human interventional study, astaxanthin and tocotrienol intake improved cognitive function.[31] Moreover, the study of the effects of vitamin B6 administration on NASH showed that there were significantly more Vit E users of in the highly effective patient group.[32] These results suggest that tocotrienols may have a synergistic effect with other vitamins and dietary factors.

In conclusion, our results suggested that orally administered T3 suppresses hepatic inflammation and fibrosis during the early stages of NASH. Further studies are needed to determine which T3 homologues are most effective and by what mechanism of action they are effective against early-stage NASH.

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Abbreviations

ALT	alanine transaminase
α-SMA	alpha smooth muscle actin
α-TTP	alpha tocopherol transfer protein
AST	aspartate aminotransferase
CDAHFD	choline deficient L-amino acid-defined high-fat diet
CHOP	C/EBP homologous protein
Col1a1	collagen type I
Col4a1	collagen type IV
ER	endoplasmic reticulum
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
H&E	hematoxylin and eosin
HPLC	high-performance liquid chromatography

MCD	mechionine-choline deficient
MMP	matrix metalloproteinase
NAFLD	non-alcohol fatty liver disease
NASH	non-alcoholic steatohepatits
PBS	phosphate buffer saline
PFA	paraformaldehyde
TC	total cholesterol
TG	triacylglycerol
TGFβ	transforming growth factor β
TIMP1	tissue inhibitor of metalloproteinase 1
TNFα	tumor necrosis factor α
Toc	tocopherol
T3	tocotrienol
Vit E	vitamin E
VLDL	very-low-density lipoprotein

Conflict of Interest

No potential conflicts of interest were disclosed.

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