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Histological evaluation of nintedanib in non-alcoholic steatohepatitis mice

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ABSTRACT

Aims: In addition to potentially progressing to either cirrhosis or hepatocellular carcinoma, non-alcoholic steatohepatitis (NASH) is currently the leading indication for liver transplantation. Nintedanib has been clinically used to treat idiopathic pulmonary fibrosis for many years, but its effects in an animal model of NASH have not been tested. The purpose of this study was to evaluate the effects of nintendanib on NASH in choline-deficient, L-amino acid-defined, high-fat diet (CDAHFD)-fed mice.

Main methods: Male C57BL/6 mice were fed a CDAHFD for 6 weeks to induce NASH with liver fibrosis, and they were administered nintedanib (60 mg/kg/day) or distilled water orally in the last 2 weeks of the feeding period. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triglyceride, and non-esterified fatty acids concentrations were measured. Serum cytokeratin 18 fragment (CK18) was detected using ELISA. Liver tissue sections from mice were stained with hematoxylin-eosin and Masson's trichrome to assess the level of steatohepatitis and fibrosis.

Key findings: CDAHFD-fed mice exhibited higher serum ALT, AST, and ALP levels compared with Control mice. A significant increase in the serum CK18 level was observed in the NASH group compared with the Control group. CDAHFD feeding also enhanced steatohepatitis and hepatic fibrosis pathological features, which were reduced after nintedanib treatment.

Significance: Nintedanib exerted anti-inflammatory and anti-fibrotic effects in CDAHFD-induced NASH mice.

1. Introduction

Aside from increasing rates of obesity and type 2 diabetes worldwide, the prevalence of non-alcoholic fatty liver disease (NAFLD), a hepatic manifestation of metabolic syndrome, also continues to increase rapidly [1]. While NAFLD is recognized as the most common cause of chronic liver disease, non-alcoholic steatohepatitis (NASH), which is an aggressive form of NAFLD, is considered to be a risk factor for cirrhosis and hepatocellular carcinoma, and it is associated with a high risk of death resulting from liver-related mortality and cardiovascular mortality [2]. In addition to hepatic steatosis, NASH is defined by any stage of liver inflammation and hepatocellular ballooning with or without progressive fibrosis [3]. Especially in patients with liver fibrosis, NASH may lead to end-stage liver disease that requires liver transplantation. Currently, NASH is the second leading indication for orthotopic liver transplantation, but it is predicted to overtake hepatitis C and become the leading aetiology for liver transplantation within the next few years [4–7].

Although there is no approved medication, several clinical trials are currently underway to support development of a drug to treat NASH patients. Because the progression of liver fibrosis is the key step to liver failure and a main driving force behind mortality in NASH patients, there has been a growing interest in pharmacological anti-fibrosis agents as potential treatments for NASH patients. These therapies can potentially reverse fibrosis or inhibit progression to severe stage [8]. Although clinical presentations of fibrotic disorders vary, shared molecular and cellular mechanisms among those diseases have been found. Thus, application of the medications that are used to treat fibrotic

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disorders to the treatment of other fibrotic diseases, especially NASH-related liver fibrosis, seems to be a logical step [9].

Nintedanib, an oral medication for idiopathic pulmonary fibrosis (IPF), is a multi-tyrosine kinase inhibitor of fibroblast growth factor receptor (FGFR), vascular endothelial growth factor receptor (VEGFR), platelet derived growth factor receptor (PDGFR), and several other kinases [10,11]. Although it is not specific for liver fibrosis, nintedanib exhibited anti-inflammatory, anti-fibrotic, and anti-angiogenic activity in an acute liver fibrogenesis mouse model [12]. Additionally, multiple phase I/II clinical trials have evaluated the safety, efficacy, and pharmacokinetics of nintedanib in patients with advanced hepatocellular carcinoma [13–15]. However, its effect in a NASH model remains unknown. To investigate histopathological features of NASH with liver fibrosis treated with nintedanib, we used a choline deficient, L-amino acid-defined, high-fat diet (CDAHFD) to rapidly develop NASH with progressive hepatic fibrosis in mice [16]. Unlike the widely used methionine choline-deficient diet model, animals fed the CDAHFD have no reduction in body weight. Consequently, the present study aimed to examine whether nintedanib affects NASH in this mouse model.

2. Materials and methods

2.1. Drug and diets

Nintedanib was purchased from LC Laboratories (Woburn, MA, USA). A normal chow diet was obtained from Oriental Yeast (Tokyo, Japan), and CDAHFD was purchased from Research Diets (New Brunswick, NJ, USA) in pellet form for a rodent diet (product No. A06071302). The CDAHFD contained 60% kcal fat, 20% kcal carbohydrate, and 20% kcal protein with 0.1% methionine, but there was no choline.

2.2. Animals and experimental protocol

Male C57BL/6 mice that were 6 weeks of age were purchased from Charles River Laboratories (Kanagawa, Japan) and randomly divided into three groups: Control, NASH, and Nintedanib groups. The Control group was fed a normal chow diet *ad libitum*, whereas the NASH and Nintedanib groups were fed the CDAHFD *ad libitum* to induce NASH with liver fibrosis. All mice remained on their diets for 4 weeks before treatment. After 4 weeks, nintedanib (60 mg/kg/day) was suspended in distilled water and administered orally by feeding needle once daily to the Nintedanib group, while they were fed the CDAHFD, for an additional 2 weeks. The Control and NASH groups received distilled water with no drug and they also continued on the same diets for an additional 2 weeks. The body weight of each mouse was monitored once weekly throughout the treatment period. At the end of the experiment, the animals were euthanized under deep anaesthesia with sevoflurane for blood and liver sample collection. All animal experiments were approved by Oita University Animal Ethics Committee (approval number: 1733001) in accordance with the guidelines for the care and use of laboratory animals.

2.3. Serum biochemical analysis

Blood samples were taken from the abdominal aorta and were allowed to clot at room temperature for 1 h. The serum was obtained after centrifugation at 1500*g* for 15 min. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), triglyceride (TG), and non-esterified fatty acids (NEFA) levels were measured using an automatic clinical analyzer (Hitachi 7180, Tokyo, Japan). The cytokeratin 18 fragment (CK18) concentration in serum was detected with a sandwich ELISA using a colorimetric commercial kit from Biomatik (Cambridge, ON, Canada), in accordance with the manufacturer's instructions.

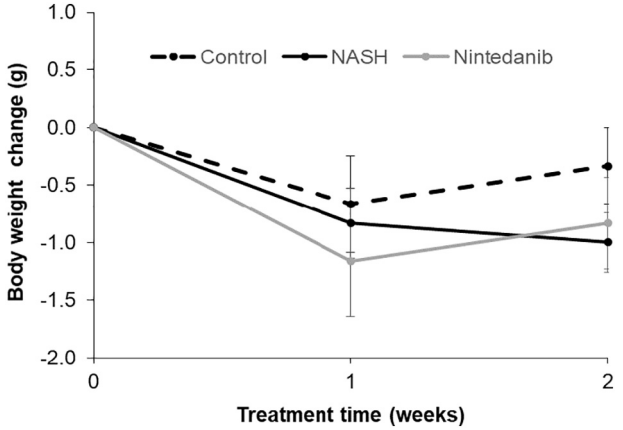

Fig. 1. Body weight changes in choline deficient, L-amino acid-defined, high-fat diet (CDAHFD)-induced NASH mice after treatment with nintedanib.
Mice were fed a normal chow diet and vehicle in the Control group, CDAHFD and vehicle in the NASH group, or CDAHFD and 60 mg/kg of nintedanib in the Nintedanib group. Body weight was monitored once a week during the 2 weeks of drug administration. Data are shown as the mean ± SEM.

2.4. Hematoxylin-eosin and Masson's trichrome staining

After collection, the liver tissues were rapidly washed in phosphate buffered saline (PBS) and fixed in 10% formalin for 24 h before they were treated with gradient ethanol dehydration and xylene. Subsequently, they were embedded in paraffin wax and cut into 5-μm thick sections using a microtome. Then, the sections were stained with hematoxylin-eosin (HE) and Masson's trichrome (MT), in accordance with the standard procedures, to visualise the severity of steatohepatitis and fibrosis, respectively.

2.5. Histological examination

The liver sections were evaluated by a pathologist who was blinded to the experiment. The pathologic features were observed under light microscope to grade the fibrosis stage (0–4) and NAFLD activity score (NAS) from steatosis (0–3), lobular inflammation (0–3), and hepatocellular ballooning (0–2), based on the criteria outlined by Kleiner et al. [17]. Quantitative analysis of the stained fibrotic areas that were positive for MT stain in the liver was conducted to estimate the mean value of MT-positive areas in each group. Three random field images of a section from each mouse were captured using an all-in-one fluorescence microscope BZ-9000 (Keyence, Osaka, Japan) and subjected to digital image analysis. At 40× magnification, the number of pixels with the predetermined color of tone (blue) were counted and the fibrotic area in each specimen was quantified using BZ-X Analyzer software (Keyence, Osaka, Japan), as described previously [18,19].

2.6. Statistical analysis

Statistical analysis was performed using PASW Statistics 18.0 software (SPSS Inc., Chicago, IL, USA). After using Kolmogorov-Smirnov test to check the normality, data were analysed using a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons and the results are presented as the mean ± standard error of the mean (SEM). Differences between experimental groups were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Effect of nintedanib on body weight and liver weight

At the end of the treatment period, the average final body weight of

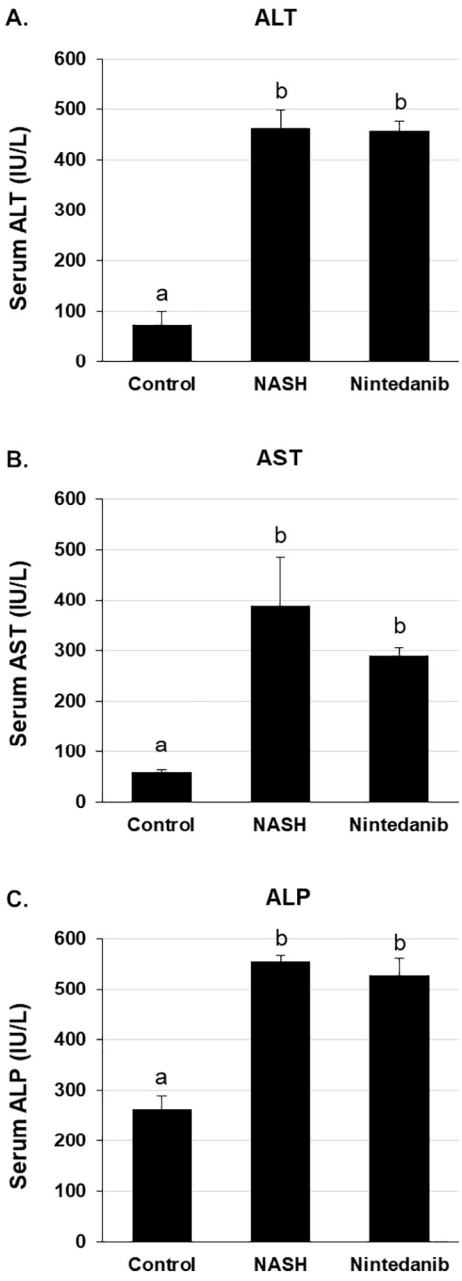

Fig. 2. Serum ALT (A), AST (B), and ALP (C) levels in normal mice or choline deficient, L-amino acid-defined, high-fat diet (CDAHFD)-induced NASH mice. Normal mice were fed a chow diet plus vehicle (Control group) and CDAHFD-induced NASH mice were given the vehicle (NASH group) or 60 mg/kg nintedanib (Nintedanib group). ALT, AST, and ALP levels were investigated in serum samples using colorimetric assays. Data in the bar graph are presented as the mean ± SEM (n = 6 in each group). Different letters indicate statistically significant differences among groups at $P < 0.05$.

the animals in Control, NASH, and Nintedanib groups were 23.17 ± 0.31, 18.83 ± 0.60, and 18.67 ± 0.42 g, respectively. The mean mouse body weight change was not significantly different among groups, indicating that nintedanib had no effect on mouse body weight (Fig. 1). Although there is no statistically significant difference in the liver weight between groups, the relative liver weights were significantly higher in both CDAHFD-fed NASH mice and Nintedanib mice than in Control mice (Table 2).

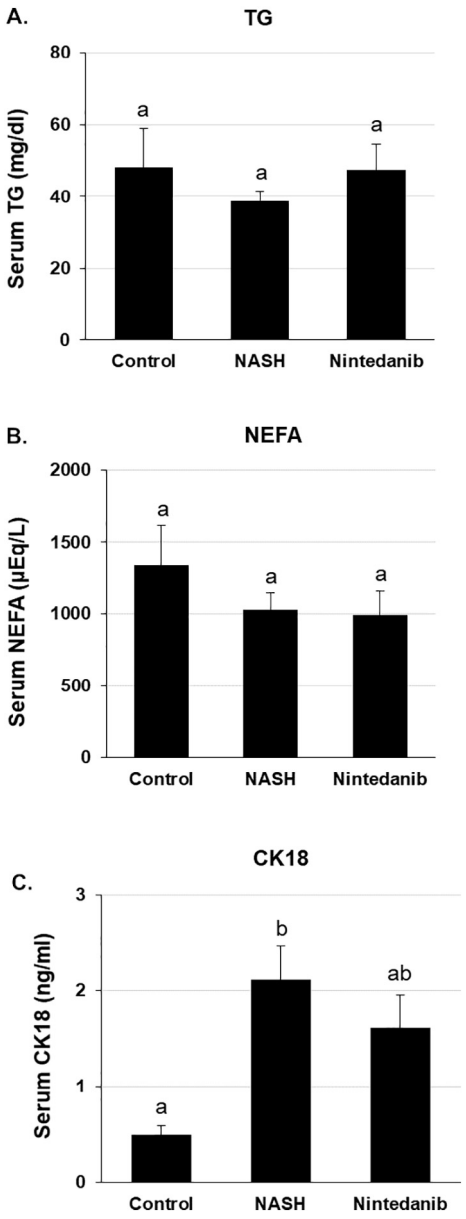

Fig. 3. Serum TG (A), NEFA (B), and CK18 (C) levels in normal mice or choline deficient, L-amino acid-defined, high-fat diet (CDAHFD)-induced NASH mice. Control mice were fed a normal chow diet, NASH mice were fed a CDAHFD, and Nintedanib mice were fed a CDAHFD plus nintedanib (60 mg/kg). The serum CK18 level was determined using colorimetric assays. Data are presented as the mean ± SEM (n = 6 in each group). Similar letters indicates no significant difference between the groups at $P < 0.05$.

3.2. Effect of nintedanib on serum parameters

Serum ALT, AST, and ALP levels were increased with the CDAHFD in the NASH and Nintedanib groups compared with the Control group (Fig. 2). Nintedanib administration had no significant effect on any of those liver enzymes. As shown in Fig. 3A, there was no significant difference in TG concentrations between the Control (48.17 ± 10.78 mg/dl), NASH (38.83 ± 2.76 mg/dl), and Nintedanib (47.33 ± 7.20 mg/dl) groups. Similarly, the NEFA values in the Control, NASH, and Nintedanib mice were 1335.83 ± 281.32, 1026.33 ± 116.55, and 993.17 ± 163.38 µEq/l, respectively, and there was no significant difference among the groups (Fig. 3B). Levels of serum CK18, a marker of hepatocyte death, in NASH mice was 4.3-fold higher compared with the Control mice ($P < 0.05$), but treatment

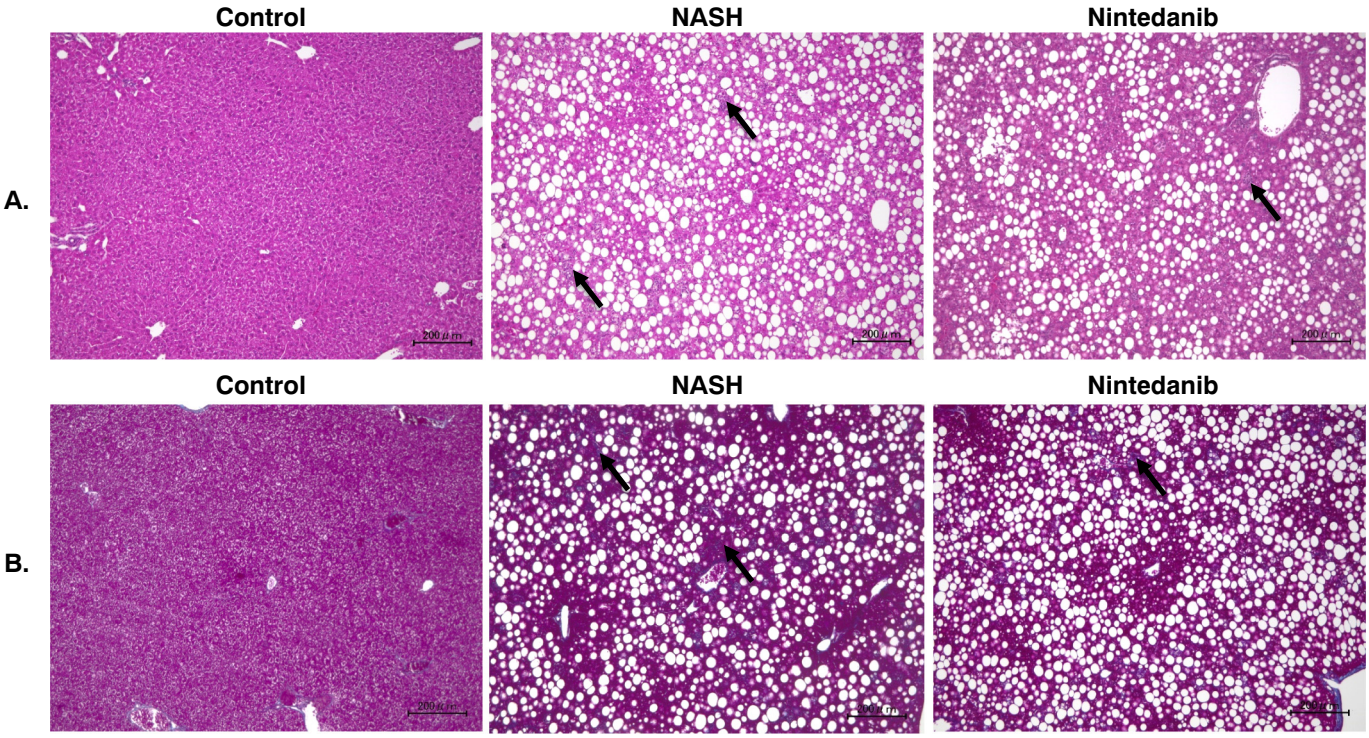

Fig. 4. Histochemical staining of liver sections from choline deficient, L-amino acid-defined, high-fat diet (CDAHFD)-fed mice with or without nintedanib administration.
Mice were maintained on a normal chow diet (Control group) or a CDAHFD (NASH and Nintedanib groups) for 4 weeks before drug administration. Thereafter, mice continued on their diet and were treated with vehicle (Control and NASH groups) or 60 mg/kg nintedanib (Nintedanib group) for an additional two weeks. Then, liver samples were collected and liver sections were stained with hematoxylin-eosin (HE) and Masson's trichrome (MT). Representative images of HE and MT staining are shown in Panels A and B, respectively. The arrows indicate the inflammatory cells in Panel A or fibrotic areas (stained in blue) in Panel B. Images were obtained at 100× magnification (scale bar = 200 μm).

with nintedanib for 2 weeks slightly reduced the CK18 levels, although the reduction was not significant (Fig. 3C).

3.3. Effect of nintedanib on histopathological changes in mouse liver

HE staining of liver sections from CDAHFD-fed mice revealed severe steatosis with inflammatory cell infiltration and few balloon cells (Fig. 4A), while MT staining of liver samples from those mice demonstrated pericellular fibrosis (Fig. 4B). Histological analysis also showed that the CDAHFD induced pathological findings that were consistent with NASH, including massive steatosis (Fig. 5A), moderate level of lobular inflammation (Fig. 5B), and mild hepatocellular ballooning (Fig. 5C), with early stage liver fibrosis (Fig. 5D) in NASH and Nintedanib mice compared with Control mice. However, mice in Nintedanib group exhibited significantly attenuated intrahepatic inflammation compared with the NASH group as confirmed by NAFLD activity score shown in Table 1. The scores of steatosis and ballooning of livers in all three groups were also shown in Table 1. Additionally, quantitative analysis displayed that the area of fibrosis was significantly increased in livers from NASH mice compared with Control mice ($P < 0.001$, Fig. 6).

4. Discussion

Simple fatty liver is benign, but NASH is a more severe form of NAFLD that includes necroinflammation and scarring in the liver, in addition to the fat accumulation. Hepatic steatosis can make the liver susceptible to injury mediated by inflammatory cytokines and oxidative stress, which promote the recruitment of inflammatory cells and hepatocyte apoptosis. This repetitive inflammatory response precedes to a continuous buildup of scar tissue that begins as fibrosis but may worsen to cirrhosis and fatal hepatic failure. Although the NASH epidemic has now spread across the globe and is correlated with the incidence of metabolic syndrome, there is no NASH-specific drug that has received approval. Based on the histological examination, the present study demonstrated for the first time that nintedanib suppresses hepatitis and liver fibrosis in a NASH mouse model that was induced by the CDAHFD.

Without using any chemical substances, a dietary NASH model involving C57BL/6 mice that received a CDAHFD is known to develop human-like NASH with steatohepatitis and hepatic fibrosis within a short time [16]. Similar to this model, feeding a CDAHFD for 6 weeks contributed to the NASH histopathological features in mice, which were confirmed by serological and histological analysis in the current study. It also resulted in the significant increases in the weights of the livers relative to body weights. Although weighing of liver after formalin fixation is a limitation of this study, previous studies [20,21] suggested that fixed liver weights could be a plausible alternative to fresh liver weights. Moreover, consistent with previous studies [22,23], the NASH animals showed increased serum liver enzyme activity, which is also comparable to those observations in NASH patients. Increases in ALT, AST, and ALP levels are usually found in the blood of patients with NASH and liver fibrosis [24–26].

In addition to the abnormalities in liver function tests observed in this model, the CK18 concentration was also significantly increased in NASH mice, which is similar to that described in humans [27,28]. CK18 is an intracellular filament protein that is largely produced during hepatocyte necrosis and apoptosis, which is a critical pathway of liver injury in NASH pathogenesis [29]. Because the CK18 level is significantly higher in patients with NASH compared with those with simple steatosis, it has been proposed as a potential non-invasive biomarker to predict NASH in multiple studies [30–34]. In this study, although nintedanib did not significantly reduce elevated liver enzyme

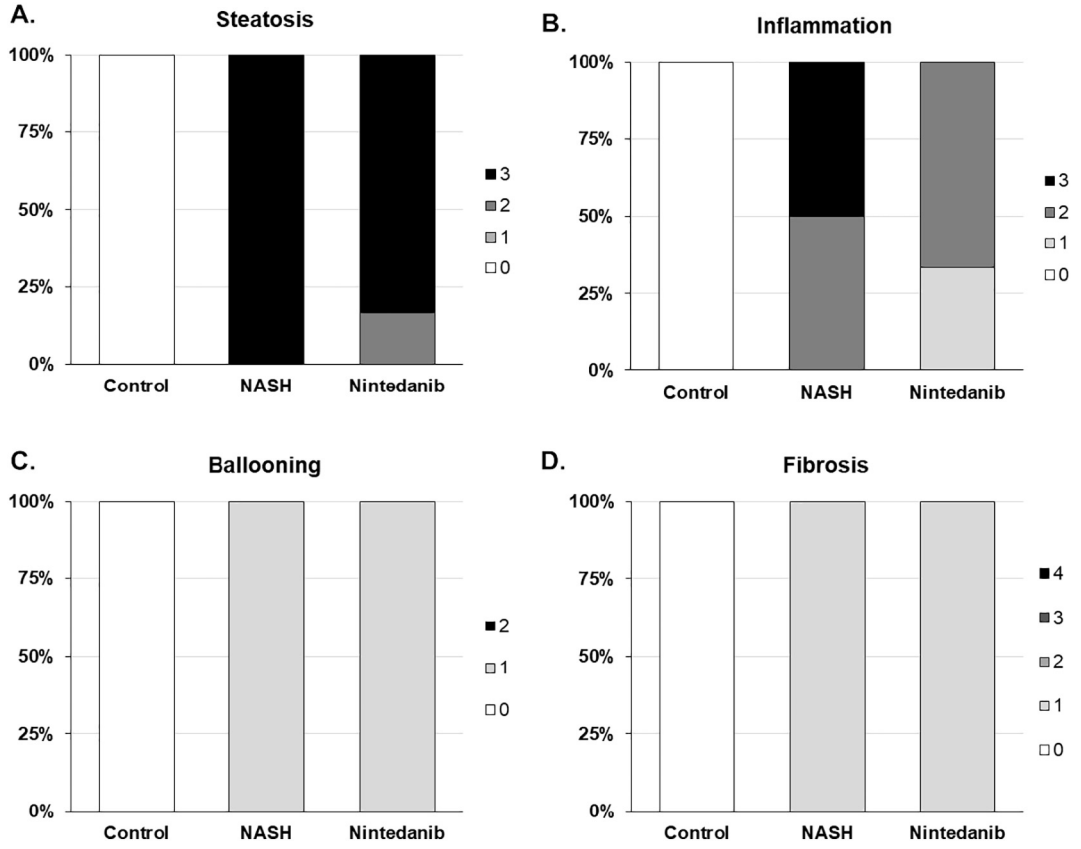

Fig. 5. Histological evaluation from liver sections of choline deficient, L-amino acid-defined, high-fat diet (CDAHFD)-fed mice with or without nintedanib treatment. The Control group was fed a normal chow diet, the NASH group was fed a CDAHFD, and the Nintedanib group was fed a CDAHFD plus 60 mg/kg of nintedanib. At the end of the study, after processing and staining with hematoxylin-eosin and Masson's trichrome, a blinded pathologist investigated the liver samples from all groups and graded the steatosis score (0–3), the inflammation score (0–3), a ballooning score, and the fibrosis stage (0–4).

Table 1
Liver weight in normal mice or choline deficient, L-amino acid-defined, high-fat diet (CDAHFD)-induced NASH mice.

	Control (N = 6)	NASH (N = 6)	Nintedanib (N = 6)
Liver weight (g)	1.40 ± 0.11	1.49 ± 0.05	1.41 ± 0.06
Liver weight/body weight (%)	6.05 ± 0.52	7.93 ± 0.30*	7.59 ± 0.32*

Control mice group were fed a chow diet, NASH mice were fed a CDAHFD, and Nintedanib mice were fed a CDAHFD with nintedanib (60 mg/kg). At the end of the experiment, livers were removed and immediately washed, then they were placed in 10% formalin. Liver weights were measured. Values are expressed as the mean ± SEM. N, sample size.

* $P < 0.05$ compared with the Control group.

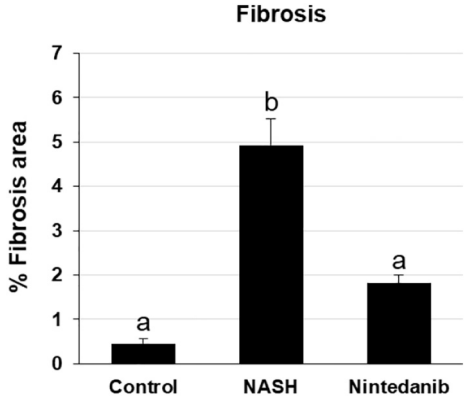

Fig. 6. The percent fibrosis per area in liver sections from choline deficient, L-amino acid-defined, high-fat diet (CDAHFD)-induced NASH mice after nintedanib treatment.
Control mice were fed a normal chow diet, whereas NASH mice and Nintedanib mice were fed a CDAHFD with or without 60 mg/kg nintedanib. Liver tissues were obtained and sectioned to quantify hepatic fibrosis (collagen deposition) by Masson's trichrome-stained areas using imaging software and the results were analysed as the average of three fields in each sections. The percentage of the fibrosis area from each group was measured. Data are shown as mean ± SEM. Different letters indicate statistically significant differences among the groups at $P < 0.001$.

serum levels after NASH development, it showed a tendency to decrease serum CK18 levels.

Nintedanib, which competitively binds the FGFR, VEGFR, and PDGFR intracellular ATP-binding sites, was originally designed as a triple angiokinase inhibitor for cancer indications [10]. As previously reported, it has a dual mechanism targeting both genetic alterations in tumor cells by directly interfering tumor cell growth and inducing apoptosis, as well as the tumor stroma by potently suppressing tumor angiogenesis [11]. Furthermore, because the pathobiology of IPF shows various similarities and links to cancer biology, nintedanib was chosen for development as a treatment for IPF and it has been explored in IPF patients [35]. Also in mouse models of lung fibrosis, regardless of the dose, preventative administration of nintedanib diminished neutrophils and lymphocytes in the bronchoalveolar lavage fluid, and reduced total lung collagen and the fibrotic score. The therapeutic regimen also showed a significant inhibitory activity of nintedanib on inflammation and fibrosis, though the effect was dependent on treatment start and duration [36]. A potential mechanism underlying its effects could be

Table 2
NAFLD activity score and fibrosis stage from liver sections of normal mice or choline deficient, L-amino acid-defined, high-fat diet (CDAHFD)-induced NASH mice.

	Control (N = 6)	NASH (N = 6)	Nintedanib (N = 6)
Steatosis	0.00 ± 0.00	3.00 ± 0.00***	2.83 ± 0.17***
Inflammation	0.00 ± 0.00	2.50 ± 0.22***	1.67 ± 0.21***,****
Ballooning	0.00 ± 0.00	1.00 ± 0.00***	1.00 ± 0.00***
NAFLD activity score	0.00 ± 0.00	6.50 ± 0.22***	5.50 ± 0.34***,****
Fibrosis	0.00 ± 0.00	1.00 ± 0.00***	1.00 ± 0.00***

Control mice group were fed a chow diet, NASH mice were fed a CDAHFD, and Nintedanib mice were fed a CDAHFD with nintedanib (60 mg/kg). Livers were collected and stained with hematoxylin-eosin and Masson's trichrome at the end of the experiment. The NAFLD activity score and fibrosis stage were assessed by a blinded pathologist. Values are expressed as the mean ± SEM. N, sample size.

*** $P < 0.001$ compared with the Control group.

* $P < 0.05$ compared with the NASH group.

due to the inhibition of discoidin domain receptor (DDR) 1 and 2, which have a major role in inflammatory and fibrotic processes [11].

In the liver, nintedanib, as a potent tyrosine kinase inhibitor, also blocks key hepatic stellate cell (HSC) receptors, including FGFR, VEGFR, and PDGFR. In chronic liver injury, activated HSCs called myofibroblasts acquire proinflammatory and fibrogenic properties by modulating the activation of lymphocytes, proliferating inflammatory chemokines, and secreting abundant extracellular matrix components [37]. While HSC activation plays a pivotal role in NASH-related hepatic fibrogenesis, activated HSCs are also a main source of growth factors such as FGF, VEGF, and PDGF [38,39]. These mediators activate HSCs in an autocrine manner and stimulate other key cell types in a paracrine manner [40,41]. Thus, although the detailed mechanism of nintedanib action in NASH with fibrosis is needed to be elucidated further in the future, simultaneous inhibition of these receptors will induce dedifferentiation of HSCs, leading to blockage of downstream signaling cascades and suppression of inflammation and fibrosis [12]. Nintedanib has also been reported to exert an anti-proliferative capacity, inhibit transformation of fibroblasts, and attenuate collagen deposition [12,36,42]. Besides, inhibitory effects of nintedanib on hepatic fibrosis and inflammation *via* stimulating M2-directed macrophage polarization were detected in a liver fibrogenesis mouse model [12]. In agreement with the results of that study, the anti-inflammatory and anti-fibrotic actions of nintedanib were also observed with the decreases of liver inflammation level and fibrosis area in the histology assessment from our study.

Although the histological scores showed that all NASH and Nintedanib mice presented the same level of fibrosis stage, the quantitative image analysis elucidated the significant decrease in fibrosis area in Nintedanib mice compared with NASH mice. Because the severity of liver fibrosis in this CDAHFD-fed mouse model increases time-dependently, 6-week experiment of this study could induce only mild perisinusoidal fibrosis (stage 1A) in this NASH model. Our preliminary data (unpublished) revealed that mice developed moderate perisinusoidal fibrosis (stage 1B) at 9 weeks after CDAHFD feeding and some mice exhibited periportal fibrosis (stage 1C) when extended to 12 weeks. However, the histological feature scoring system for fibrosis stage comprises 4 stages from no fibrosis (stage 0) to cirrhosis (stage 4) with subclassification of stage 1 into that 3 categories. The maximum level of hepatic fibrosis we found in the mice from this study is the minimum levels of the present fibrosis based on these criteria. Thus, nintedanib could reduce liver fibrosis but could not fully reverse all liver scarring to reach the stage 0 according to the grading system. Perhaps an induction period of > 6 weeks is required to induce a higher stage of hepatic fibrosis in the CDAHFD-fed mice and to distinguish the fibrosis stages after treatment.

Apart from that limitation in our study, increases in liver enzyme levels, such as ALT, AST, and ALP, were reported in patients receiving nintedanib in some clinical studies [43–45], although changes in these liver enzyme levels were not associated with clinical manifestations of liver damage and were reversible after dose reduction. Further studies are required to clarify the molecular mechanism behind its effect. The investigation of inflammatory cytokines, fibrosis markers, and other mediators involved in NASH and liver fibrosis should be carried out to confirm the therapeutic effects of nintedanib on fibrotic NASH.

5. Conclusions

Based on the histological assessment, this preclinical study has shown that liver inflammation and fibrosis are significantly attenuated by nintedanib in a mouse model of NASH that was induced by a CDAHFD.

Declaration of Competing Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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