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Original Article

Water Extract of *Vitis coignetiae* Pulliat Leaves Attenuates Oxidative Stress and Inflammation in Progressive NASH Rats

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This study aimed to investigate the therapeutic effects of the water extract of leaves of *Vitis coignetiae* Pulliat (VCPL) on nonalcoholic steatohepatitis (NASH) with advanced fibrosis, as our previous study exhibited its preventive effect on NASH. The NASH animal model [PCT/JP2007/52477] was prepared by loading recurrent and intermittent hypoxemia stress to a rat with fatty liver, which resembled the condition occurring in patients with obstructive sleep apnea (OSA) and fatty liver, who have a high incidence of NASH. Intermittent hypoxemia stress is regarded as a condition similar to warm ischemia followed by re-oxygenation, which induces oxidative stress (OS). The daily 100 or 300 mg/kg VCPL administrations were performed for 3 weeks perorally beginning at the time of detection of advanced liver fibrosis. The therapeutic efficacy of VCPL on NASH was demonstrated by the reduction of the leakage of hepato-biliary enzymes and the amelioration of liver fibrosis. The OS elevation in NASH rats was measured based on the derivation of reactive oxygen species from liver mitochondrial energy metabolism and on the decrease in plasma SOD-like activity. The aggravation of inflammatory responses was demonstrated by the neutrophil infiltration (elevated myeloperoxidase activity) and the progression of fibrosis in the livers of NASH rats. In addition, the NASH rats without VCPL treatment also exhibited activation of nuclear factor-κB, a key factor in the link between oxidative stress and inflammation. All of these changes were reduced dose-dependently by the VCPL administration. These findings indicate that VCPL may improve hepatic fibrosis or at least suppress the progression of NASH, by breaking the crosstalk between OS and inflammation.

Key words: non-alcoholic steatohepatitis, antioxidative, oxidative stress, anti-inflammation, *Vitis coignetiae* Pulliat

Nonalcoholic steatohepatitis (NASH), one of most common liver diseases, is characterized by necroinflammatory activity, with nonspecific inflammatory infiltrates, hepatocyte ballooning with Mallory's hyaline and occasional fibrosis [1]. Although the etiology of NASH remains unclear, the "two-hit theory" describes the most widely accepted mechanism for the progression of NASH [2].

Oxidative stress (OS) is regarded as the "second hit" in the development of NASH, following the "first hit" of hepatic fat accumulation. Mitochondrial dysfunction in the NASH liver causes and maintains overproduction of reactive oxygen species (ROS) [3, 4]. This excessive ROS activates nuclear factor-

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kappa B (NF-κB), induces inflammatory cytokines and a variety of other inflammatory factors, and stimulates the hepatic stellate cells (HSCs), which play an important role in fibrogenesis [4, 5]. A previous clinical study demonstrated that the long-term administrations of vitamin E and vitamin C improved the fibrosis in NASH patients [6]. Thus, the regulation of oxidative stress could potentially contribute to the treatment of progressive NASH. However, the optimum treatment for NASH remains to be established.

It is widely recognized that grapes confer health benefits through their potent antioxidative abilities, which are believed to play a major role in the so-called "French paradox." *Vitis coignetiae* Pulliat, or Yamabudo in Japanese, is a Japanese wild grape species, and shows very high levels of polyphenols such as anthocyanins in both the fruit and peel, compared with other grape species [7]. As such, it has been widely been utilized in drinks such as juice and wine. Several biological properties have been attributed to *Vitis coignetiae* Pulliat, including high free-radical-scavenging activity and resistance to photodecomposition of anthocyanin pigments [8]. We focused our attention on leaves of *Vitis coignetiae* Pulliat (VCPL), because the same functional constituents found in the fruit and the peel are also present in the leaves, as they produce and provide various components to plants. The safety of VCPL as a food is secured, because, the traditional custom of serving leaves of Vitis coignetiae Pulliat as an edible materials exists in Hiruzen district, Okayama.

It has been reported that the rate of NASH development is 7-fold higher in patients with obstructive sleep apnea (OSA) and fatty liver, compared to those with fatty liver, so the association of OSA and NASH has been the target of growing attention [9, 10]. Here, we used a clinically relevant rat model of NASH (patent application no. PCT/JP2007/52477). Rats were fed a CDHF diet continuously for 4 weeks to induce steatosis, and then exposed to recurrent and intermittent hypoxemia stress to induce liver dysfunction [11] within a relatively short period of time. Recurrent and intermittent hypoxemia is considered a condition similar to warm ischemia-reperfusion, which is accepted as a cause of oxidative stress in patients with OSA [9, 10].

Using this morbid model of NASH, we previously demonstrated that VCPL has a preventive effect on the progression of NASH through its high antioxidative activity and its ability to attenuate the induction of cytochrome P450 2E1 (CYP2E1) [12] for which a role in ROS production, oxidative stress, and lipid peroxidation has been implicated. In the present study, we aimed to investigate the therapeutic effect of VCPL on NASH with liver fibrosis, to focus on the OS from mitochondria metabolism and inflammatory responses.

Materials and Methods

Preparation of VCPL water extracts. The leaves were collected in 3 seasons (July, September, and the end of October) at Hiruzen Highland (Maniwa, Okayama, Japan) where the improved variety of *Vitis coigunetiae* Pulliat is cultivated on a large scale and is one of the special local products, as there is suitable for its growth The leaves gathered in July and September were green, and those gathered in October were red. The water extracts were prepared from the VCPL from all 3 seasons as mentioned below, and their antioxidative abilities were found to be very strong (data not shown) and similar each other. In this study, we used the water extract from the red leaves gathered at the end of October after post-harvest for the animal experiment, because their water extract was superior by its beautiful red color and by its mellow aroma like a red wine, compared to those from green leaves of July and September. Besides the post-harvest VCPL is advantageous as a material of functional foods, there is not the influence on fruit harvest.

The leaves were washed, freeze-dried, and powdered. The powder (50 g) was extracted by distilled water (1 L) for 10 min at 4℃. The extract was filtered and then the solvent was evaporated to dryness under reduced pressure in a rotary evaporator. The residual extract was freeze-dried to powder, and used for the study. To check the safety of VCPL water extracts, we executed the analyses for the determination of pesticide residues and heavy metals such as cadmium, lead, arsenic and mercury in a freeze-dried preparation. All of these contents in the freeze-drying preparation of the water extracts of VCPL were either under the detection limits or under the maximum residue limits for pesticides and the standards for the tolerance of heavy metals (data not shown) for

which standards have been legally established with regard to food ingredients pursuant to the Food sanitation Act. After confirming the safety of VCPL with respect to pesticide residues and heavy metals, the water extract of VCPL was used for an animal experiment.

Animals and experimental design. Six-week-old male Wistar rats were purchased from Shimizu Animal Co. (Kyoto, Japan) and maintained at the Okayama University animal facility in accordance with the Okayama University Guidelines on the Care and Use of Laboratory Animals. Rats were housed in individual cages in air-conditioned rooms with a controlled 12-h light-dark cycle. The rats were allowed to acclimate to the animal facility and were provided free access to diets and water *ad libitum*.

The NASH rat model [PCT/JP2007/52477] was prepared by our method [11]. The rats were fed either a standard chow diet for rodents (MF laboratory chow; Oriental Yeast Co., Tokyo, Japan) (the Control group; n = 5), or a choline-deficient high-fat (CDHF) diet (Oriental Yeast Co., Tokyo, Japan) (n = 30) throughout the experimental period of 13 weeks. Fatty liver was formed by the feeding with CDHF for 4 weeks. Then rats with fatty liver were divided into 3 groups to equalize their body weight. Beginning in the fifth week, the fatty liver rats were treated with intraperitoneal injection of 40 mg/kg of sodium nitrite ($NaNO_2$) (Nacalai Tesque Inc., Kyoto, Japan) every day to induce methemoglobinemia (intermittent hypoxemia stress) for 9 weeks (NASH group; n = 10). In our previous study, we demonstrated that the advanced fibrosis of NASH appeared 6 weeks after the injection of $NaNO_2$ [11]. Thus, the oral VCPL administration was performed during the last 3 weeks, with a dose of 100 mg/kg/day VCPL in the NASH+VCPL 100 group (n = 10), and 300 mg/kg/day VCPL in the NASH+VPCL 300 group (n = 10).

At the end of the 13-week experimental period, the rats were sacrificed under light ether anesthesia, blood was collected from the inferior vena cava for plasma analysis, and the livers were resected for histopathological and biochemical analyses to determine OS-related injuries and the VCPL efficacy, and to investigate the mechanism.

Leakages of hepato-biliary enzymes. Plasma AST and ALT activities were measured using a commercially available kit, Transaminase CII-test Wako (Wako Pure Chemicals, Osaka, Japan). The activity of alkaline phosphatase (ALP) was estimated from the released amounts of *p*-nitrophenol as measured by the absorbance at 410 nm.

Liver triglycerides. The supernatant fraction obtained by the centrifugation of 5% liver homogenate with 0.25 M sucrose Tris-HCl buffer containing 0.1 M potassium chloride (KCl) at 200 g for 10 min was submitted for determination of liver triglycerides using a quantitative assay kit for triglycerides (Wako Pure Chemical Industries, Osaka, Japan).

Liver histopathology. Liver tissues were fixed in 10% formalin, embedded in paraffin and sectioned at a thickness of 4 μm. The liver sections were stained with hematoxylin-eosin and Masson's trichrome staining methods. Evaluations of histopathological changes such as fat accumulation and fibrosis in the liver were performed by a microscopic observation and captured images, using a digital light microscope (Olympus, Tokyo, Japan). The blue staining parts in the liver tissue sections stained with Masson's trichrome stain are the collagen-rich fibrotic regions and the connective tissues. To assess the liver fibrosis, the blue parts in the digital images of preparations with Masson's trichrome stain were measured using image analysis software, WinROOF (Mitani Corp., Fukui, Japan).

Radical trapping studies. The antioxidative activity of rat plasma was estimated according to the ability to scavenge superoxide anion ($O_2^{\bullet-}$), which in turn was determined with an electron spin resonance (ESR) spectrometer (JES-RE1X JEOL RE-1X; JEOL, Tokyo, Japan) connected to a data analyzer (WIN-RAD; Radical Research Inc., Tokyo, Japan) using 5,5-dimethyl-1-pyrroline-1-oxide (DMPO) (Labotech Co., Tokyo, Japan) as a radical-trapping reagent [13]. A 45 μl aliquot of a 5% plasma sample solution containing 287.5 mM DMPO, 500 μM hypoxanthine, 963 μM diethylenetriaminepenta-acetic acid (DETAPAC), 5% dimethyl sulfoxide (DMSO) and 0.1 U xanthine oxidase in a 0.1 M phosphate buffer solution (PBS), pH 7.8, was placed in a capillary glass tube (i.d. 1 mm), which in turn was introduced into a quartz tube (i.d. 4 mm, o.d. 5 mm; LST-5HG; Labotech, Tokyo, Japan). The recording of the ESR spectrum of the sample was initiated 45 sec after the xanthine oxidase addition, at room temperature. The instrument conditions were as follows: about 9.4 GHz with 100-kHz modulation fre-

quency, central field ± sweep width of 335.3 ± 5 mT, microwave power of 8 mW, field modulation width of 0.079 mT, time constant of 0.1 sec, sweep speed of 5 mT/min. The magnetic field was calculated from the splitting of divalent manganese (Mn^{2+}), in which the distance from the third to fourth signal is 8.69 mT.

The SOD-like activity was calculated from the ratio of the 1st signal of the spectrum of the spin adducts of DMPO and $O_2^{\bullet-}$ (DMPO-OOH)/the 3rd signal of Mn^{2+}, by fitting to the calibration curve drawn with standard Cu, Zn-superoxide dismutase (Cu, Zn-SOD).

ROS derivation from liver mitochondria. To examine the ROS derivation from liver mitochondria, a 35 μL aliquot (500 μg protein) of mitochondrial suspension in 0.25 M sucrose Tris-HCl buffer containing 0.1 M KCl was mixed with 25 μL of substrate solution containing 0.4% dodecyl maltoside, 40 mM sodium glutamate, 40 mM disodium malate and 800 mM disodium succinate in 50 mM potassium phosphate buffer, pH 7.4, 20 μL of 4.6 M DMPO and finally 1.0 mM NADH (Oriental Yeast Co. Osaka, Japan), and then was incubated at 37℃. After 5 min incubation following the NADPH addition, a 45 μL aliquot of the above-mentioned mixture was placed into a capillary glass tube, which in turn was introduced into a quartz tube and subjected to investigate the mitochondrial ROS derivation, by the spin trapping method of ESR described above.

In this measurement, the characteristic ESR spectrum of the spin adducts of DMPO and hydroxyl radicals (DMPO-OH) was detected. Therefore, the ROS derivation was estimated by the ratio of the 2nd signal intensity of the DMPO-OH spectrum/the 3rd signal of Mn^{2+}.

Myeloperoxidase activity. Myeloperoxidase (MPO) activity was determined according to the method of Schneider *et al.* [14] with minor modifications. The samples of 25% liver homogenate in 0.25M sucrose Tris-HCl buffer (pH 7.4) were diluted to 5% with 10 mM citrate buffer (pH 5.0) containing 0.22% cetyltrimethylammonium chloride (CTAC; Wako Pure Chemicals, Osaka, Japan) and 0.3 M sucrose, and centrifugation was performed at 6,500 × g for 20 min at 4℃, and the supernatant was used for the sample to determine MPO activity, the index for the neutrophil infiltration into liver tissue. Aliquots of 90 μL of each liver supernatant (10 mg protein/mL in 10 mM citrate buffer, pH 5.0) were pipetted into 4 wells. Thirty μL of MPO substrate solution containing 6 mM 3, 3', 5, 5'-tetramethylbenzidene dihydrochloride (TMBZ), 120 μM resorcinol, and 4.4 mM H_2O_2 in distilled water was added to each well, and the reaction was stopped after 2 min with 120 μL of cold stop solution, and the MPO activity was measured the absorbance at 450 nm. As an additional control, 90 μL of dilution buffer (without liver extract) was pipetted into 4 wells, followed by the addition of 30 μL of substrate buffer, and then, after 2 min, 120 μL of stop solution. No color reaction was observed in these control wells. The MPO activity of the liver sample was calculated by subtracting the mean background absorbance and is expressed as the change in optical density per minute.

Nuclear extracts and Western blot analyses. The nuclear fractions were obtained by the precipitation of 25% liver homogenate with 0.25 M sucrose Tris-HCl buffer (pH 7.4), which was obtained by the centrifugation of 700 × g for 10 min at 4℃. Then, this precipitation was resuspended in 50 mM HEPES buffer (pH 7.4) containing 0.1 M KCl, 3 mM magnesium chloride, 1 mM ethylenediaminetetraacetic acid, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/mL pepstatin A, 5 μg/mL leapeptin and 2 μg/mL aprotinin, and centrifuged at 22,000 × g for 20 min at 4℃. The supernatant was then used for the analysis of nuclear proteins. The nuclear proteins were diluted to 6 mg/mL, then mixed with sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 25% glycerol, 2% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 0.01% bromophenol blue) and denatured at 95℃ for 5 min. The samples (in 30 μg protein/10 μL) were separated on SDS-12.5% polyacrylamide gel (Bio-Rad Laboratories Inc., Hercules, CA, USA), and then transferred to a polyvinylidene fluoride (PVDF) membrane using a trans-blot apparatus (Bio-Rad Laboratories Inc.). The membranes were blocked in 5% nonfat milk dissolved in TBS-T buffer (25 mM Tris-HCl buffer, pH 7.4, containing 0.15 M sodium chloride (NaCl) and 0.1% Tween20) for 1 h at room temperature, and incubated with primary antibodies (mouse monoclonal anti-rat NF-κB p65 (1 : 1000 dilution) and rabbit polyclonal anti-rat Histone H1 (1 : 200 dilution); Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4℃, and then incubated with secondary antibodies (goat anti-mouse antibody or goat anti-rabbit antibody (1 : 5000 dilution); Santa Cruz Bio-

technology) for 0.5 h at room temperature. Visualization was performed with Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology). The quantity of NF-κB was corrected by that of Histone H1 in the same preparation.

Statistical analysis. Quantitative data were expressed as the mean ± SEM. The statistical analysis was performed using a Student's *t*-test or one-way ANOVA with Tukey. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of VCPL on body weights and liver indices. The body weights of rats in the NASH, NASH+VCPL100, and NASH+VCPL300 groups, all of which were fed a CDHF diet, significantly decreased compared to those of the Control rats fed a standard chow diet. There were no differences in body weights among the NASH, NASH+VCPL100, and NASH+VCPL300 groups (Table 1). Calculating from the feed consumption, there were also no differences in the calorie intakes of the NASH, NASH+VCPL100, and NASH+VCPL300 groups (319.3± 64.7, 323.5 ± 68.8 and 317.3 ± 65.5 cal/kg/day, respectively), while the caloric intake of the Control group (365.3 ± 79.7 cal/kg/day) was significantly higher than those of each of the 3 experimental groups.

Compared to the Control rats, there were decreases in the wet liver weights, and increases in the liver indices in NASH rats, as the weight decrease of the body counteracted that of the liver. Further, the body weights, the wet liver weights and the liver indices were 248.5 ± 9.7 g and 12.2 ± 1.3 g and 4.9 ± 0.8%, respectively, in the rats (n = 4) fed only CDHF without intraperitoneal $NaNO_2$ injections. The VCPL administration induced significantly greater decreases of the wet liver weights and the liver indices ($p <$ 0.01) in NASH rats. Incidentally, the rats administered VCPL did not show any toxic responses such as body weight changes, allergic responses like skin rash, or other organ injuries.

Effects of VCPL on leakages of hepato-biliary enzymes. The activities of ALT, AST and ALP in plasma were significantly ($p<0.01$) elevated in the NASH group compared to the Control group. The VCPL administration significantly ($p<0.05$) reduced the ALT elevation (Table 1). The VCPL administration resulted in a tendency to reduce the AST and ALP activities of the NASH group, however the differences of activities between the groups without and with the VCPL administration, were not statistically significant.

Histopathological changes in liver. Histological examination of the livers of NASH rats prepared by feeding with CDHF for 13 weeks in addition to $NaNO_2$ treatment during the last 9 weeks revealed marked steatohepatitis (Fig 1. B, F), compared with the Control group (Fig. 1A, E). Fatty change stained white was mostly a macrovesicular type, involving all zones of the lobe. Inflammatory infiltrates consisted of foci of acute and chronic inflammatory cells randomly distributed in the lobe. Apparent fibrosis of the liver was observed in all the groups except for the liver preparation of the Control group, as the collagen-rich fibrotic regions and the connective tissue are stained

Table 1 Effects of VCPL on body weight, liver indices and leakages of hepato-biliary enzymes

		NASH		
	Control	0	100 VCPL (mg/kg/day, p.o.)	300
Body weight (g)	414 ± 9.3	225 ± 8.1††	231 ± 8.6	233 ± 7.2
Wet liver weight (g)	10 ± 0.4	7.9 ± 0.9	10 ± 0.9	9.9 ± 0.9
Liver index (%)	2.4 ± 0.1	3.4 ± 0.3†	4.5 ± 0.3**	4.2 ± 0.2**
Biochemical marker (plasma)				
ALT (U/mL)	9.8 ± 0.8	23.1 ± 3.5††	12.1 ± 2.0*	14.4 ± 1.3*
AST (U/mL)	101.1 ± 8.3	422.0 ± 19.3††	420.0 ± 35.6	346.0 ± 10.0
ALP (Released *p*-nitrophenol, mM)	0.21 ± 0.06	3.37 ± 0.21††	2.41 ± 0.64	2.18 ± 0.37

Each value denotes the mean ± SEM for 6-8 rats except for the Control group (n = 5). The liver index (%) was calculated as the wet liver weight/body weight ×100. $^{\dagger}p < 0.05$, $^{\dagger\dagger}p < 0.01$ vs. Control. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. NASH.

blue by Masson's trichrome stain. The VCPL administration did not change the level of macrovesicular steatosis (Fig. 1C, D) and abated liver fibrosis (Fig. 1G, H).

The fat accumulation in hepatocytes of NASH rats was also quantified by the triglyceride contents, which increased significantly ($p<0.01$) compared to those of Control rats. The VCPL administration at the doses of 100 mg and 300 mg/kg/day had no effect on triglyceride contents in the liver of NASH rats (Fig. 1I).

As the collagen-rich fibrotic regions and the connective tissue are stained blue by Masson's trichrome staining, there were detected the liver fibrosis as the significant increase of blue staining area in the liver tissue sections of NASH rats. This significant fibrosis was ameliorated by VCPL administration (Fig. 1J).

Effect of VCPL on plasma SOD-like activity. The SOD-like activity in plasma was significantly ($p<0.05$) reduced in the NASH group compared to the Control group (Fig. 2). The reduced plasma SOD-like activity in NASH group was significantly ($p<0.05$) elevated by VCPL administration.

Effect of VCPL on ROS derivation from liver mitochondria. The liver mitochondria of ROS derivation could be detected by signals from the DMPO-OH spin adducts in our ESR analysis. The signal intensities were significantly ($p<0.01$) elevated in the NASH group compared to the Control group

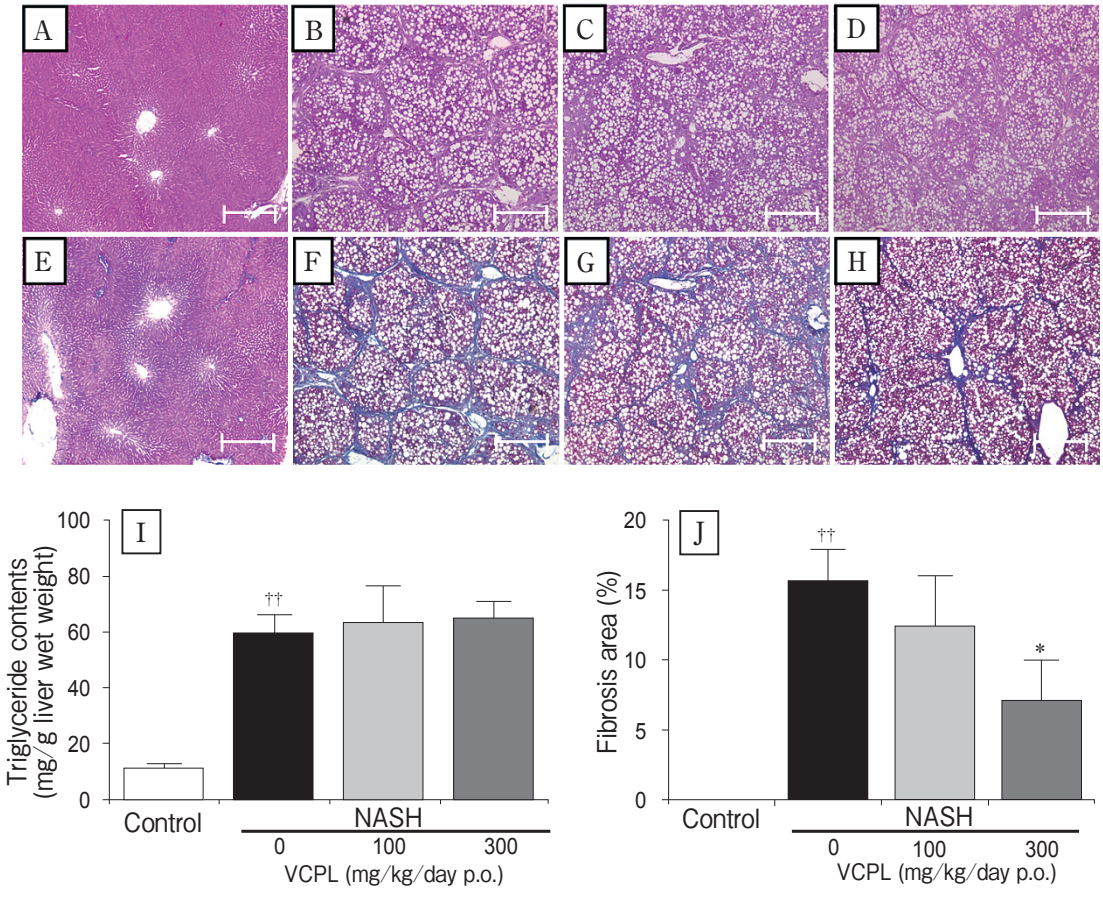

Fig. 1 Hepatic pathology in liver tissues of the experimental rats. Liver tissue structure was assessed in the hematoxylin and eosin staining preparation of Control (**A**), NASH (**B**), NASH+VCPL100 mg/kg (**C**) and NASH+VCPL300 mg/kg (**D**) rats. White regions show either the vascular cavity or fat accumulated hepatocytes. Blue regions show collagen-rich parts, either the fibrosis or the connective tissue around vessels, in Masson's trichrome staining preparations of Control (**E**), NASH (**F**), NASH+VCPL100 mg/kg (**G**) and NASH+VCPL300 mg/kg (**H**) rats. Scale bar = 500 μm. The quantitative data of adiposity and fibril formation in the liver are shown at **I** and **J**. Each value denotes the mean ± SEM for 6-8 rats except for the Control group (n = 5). $^{\dagger\dagger}p < 0.01$ vs. Control, $^{*}p < 0.05$ vs. NASH.

(Fig. 3). The high signal intensities in the NASH group were significantly ($p<0.05$, $p<0.01$) reduced by the VCPL administration in a dose-dependent manner.

Effect of VCPL on MPO activity in the liver. MPO activity in the liver as an indicator of neutrophil infiltration was significantly ($p<0.01$) elevated in the NASH group compared to the Control group (Fig. 4). The elevated MPO activities in the NASH group were significantly ($p<0.05$, $p<0.01$) reduced by the VCPL administration in a dose-dependent manner.

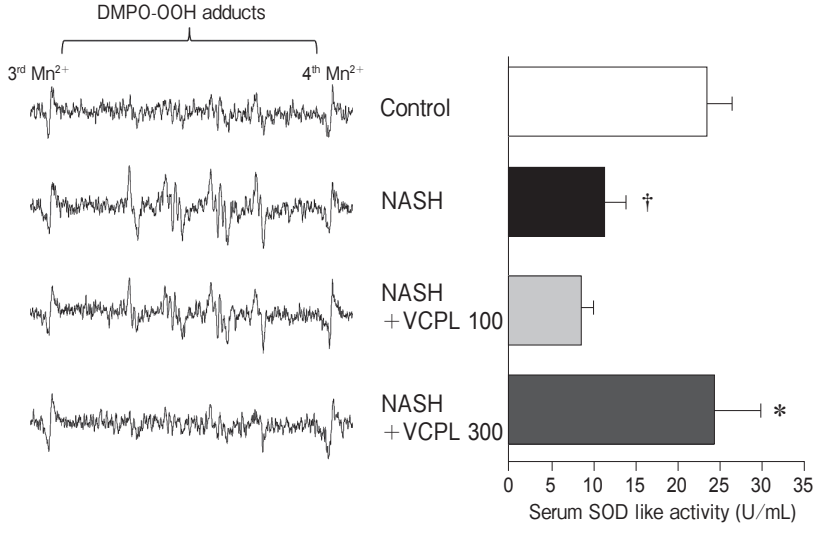

Fig. 2 Effects of VCPL on plasma SOD-like activity of rats. ESR spectra of DMPO-OOH spin adducts were detected by the mixtures of $O2^{\cdot-}$ generating systems with the plasma samples of Control, NASH, NASH+VCPL100 and NASH+VCPL300 rats denoted at the right. Each value denotes the mean ± SEM for 6-8 rats except for Control group (n = 5). $^{\dagger}p < 0.05$ vs. Control. $^{*}p < 0.05$ vs. NASH.

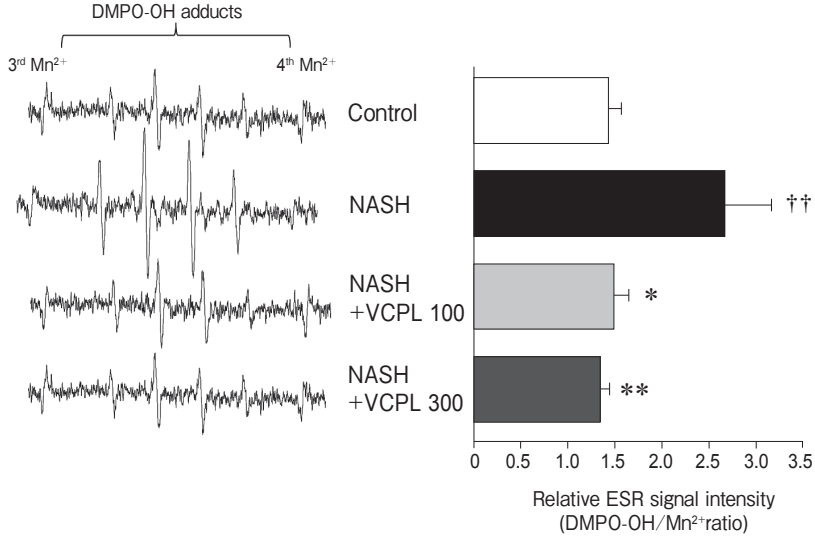

Fig. 3 Effects of VCPL on the mitochondrial ROS derivation from rat liver. ESR spectra of DMPO-OH spin adducts were detected by the mixtures of the mitochondrial respiratory substrates with the liver mitochondrial preparations of Control, NASH, NASH+VCPL100 and NASH+VCPL300 rats denoted at the right. Each value denotes the mean ± SEM for 6-8 rats except for Control group (n = 5). $^{\dagger\dagger}p < 0.01$ vs. Control. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. NASH.

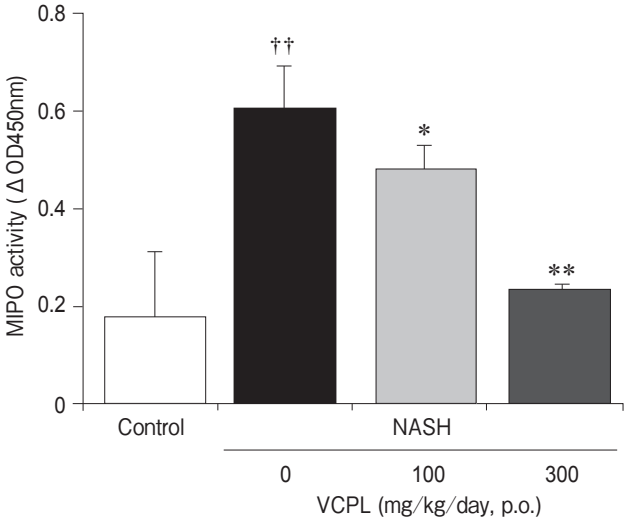

Fig. 4 Effects of VCPL on the myeloperoxidase activities of rat liver. Myeloperoxidase (MPO) activity as a marker of neutrophil infiltration was detected with absorbance at 540nm. Each value denotes the mean ± SEM for 6-8 rats except for Control group (n = 5). $^{\dagger\dagger}p < 0.01$ vs. Control, $^{*}p < 0.05$, $^{**}p < 0.01$ vs. NASH.

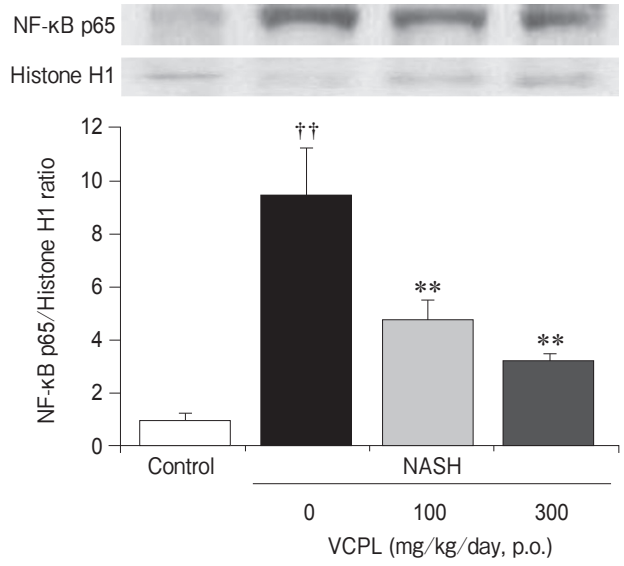

Fig. 5 Effects of VCPL on NF-κB activation in hepatocytes of rats. By the activation, NF-κB is imported into the nuclear fraction. The levels of NF-κB p65 and Histone H1 in the nuclear fraction of hepatocytes were measured by Western blot. The band density of NF-κB was compensated by the respective Histone H1, and further the data were shown on the basis of Control group. Each value denotes the mean ± SEM for 6-8 rats except for Control group (n = 5). $^{\dagger\dagger}p < 0.01$ vs. Control, $^{**}p < 0.01$ vs. NASH.

Effect of VCPL on the expression of NF-κB p65 protein. The activation of NF-κB was measured according to the amount of NF-κB in the nucleus by Western blotting analysis of the nuclear protein of rat liver. Significant NF-κB activation ($p < 0.01$) was demonstrated by the increased NF-κB p65 band in the NASH group compared to that in the Control group (Fig. 5). The increased NF-κB incorporation into the nuclei in the hepatocytes of NASH group was significantly ($p < 0.01$) decreased by VCPL administration.

Discussion

This is the first study to show that VCPL is able not only to prevent the development of NASH, but also to ameliorate the condition once developed. The present study demonstrated that oral VCPL administration for NASH model rats with advanced liver fibrosis could lessen the leakages of hepato-biliary enzymes (Table 1), but also could ameliorate the condition once developed. Further, VCPL was shown to be able to correct the oxidative stress enhancement in NASH rats, to regain the decrease of plasma antioxidative abilities and to ameliorate the increase of mitochondrial ROS derivation.

The water extracts from green leaves were also suggested to be effective, because there was no significant difference between the abilities of red and green leaves to scavenge oxygen free radicals *in vitro* (data not shown). In contrast, the water extracts from red leaves post-harvest were superior in palatability and there was no influence on the fruit yield. Therefore, in the present study we used the red leaves post-harvest.

Compared to those of the Control group, the body weights decreased in the NASH groups fed a CDHF diet, independent of VCPL administration. This decrease would have resulted from a decrease in caloric intake, since the CDHF diet was in paste form and more difficult to eat than a standard solid diet. Thus, the VCPL can be considered safe as a food for human consumption, since its administration did not induce a change in the body weights of NASH rats or any toxic responses such as allergy or other organ injuries, including kidney injuries (data not shown), in addition to its having been a traditional food in the Hiruzen district of Okayama since ancient times.

In our NASH model, the pathologic conditions were ameliorated by the dissolution of choline deficiency, because the fatty liver formation was accelerated by a CDHF diet, to prepare NASH for a short term. However, the choline deficiency was not relieved by the VCPL administrations, as its content in VCPL was 0.6 mg/g freeze-dried preparation. The 300 mg/kg/day VCPL administration supplies choline at a dose of 0.018 mg/kg/day, while the standard diet contains 2 mg/g choline.

A hydrophilic reagent, $NaNO_2$, was injected intraperitoneally, to provoke methemoglobinemia and decay the oxygen transport ability, which induced the intermittent hypoxemia. A direct reaction between $NaNO_2$ and VCPL was hardly expected, because the interval of both administrations was 3 h. Rather, it was suggested that VCPL would improve methemoglobinemia by increasing the antioxidative defense abilities, such as the elevation of SOD-like activity (Fig. 2), which in turn would increase the reduced glutathione.

Oxidative stress-related injury has been considered a likely basal process in the progression of hepatocellular damage, inflammation, and fibrosis [15–18]. Although there have been many reports of food ingredients with antioxidative activities that can prevent clinical progression of NASH, including green tea extracts [19], fermented green tea extracts [20] and N-acetylcysteine [21], these ingredients have not been shown to have effects on treated progressive NASH.

In comparison with Control rats, the liver indices exhibited hepatomegaly ($4.9 \pm 0.8\%$) in rats only fed CDHF without $NaNO_2$ injections that corresponded to the fatty liver status without severe fibrosis (data not shown). Compared to the liver index of $4.9 \pm 0.6\%$ described above, that of NASH rats ($3.4 \pm 0.3\%$; Table 1) indicated liver atrophy. VCPL administration alleviated the liver atrophy that indicated the lessened fibrosis, compared with NASH rats (Table 1, Fig. 1). Therefore, these findings indicated that VCPL could improve NASH by inhibition of "second hits".

In addition, previous studies identified the pigments in the fruit and peel of *Vitis coignetiae* Pulliat as mostly malvidin-3, 5-diglucoside and malvidin-3, 5-diglucoside-coumarate [22]. Some studies have defined anthocyanins as having remarkable antioxidative and oxygen radical-scavenging properties [23, 24]. Previous studies on leaves from European grapes (*Vitis vinifera* L.) showed antihyperglycemic effects in diabetic rats [25, 26] and hepatoprotective activities [27, 28]. These findings would thus support the finding in this study that the anthocyanins in VCPL also conferred hepatoprotective effects in NASH rats.

MPO is a well-known enzyme associated with the generation of reactive oxygen/nitrogen species and inflammation that lead to the progression of human NASH [29]. This study demonstrated that MPO activity was significantly elevated in the livers of rats with NASH (Fig. 4). In addition, this study showed an increase in mitochondrial ROS derivation in the livers of NASH rats (Fig. 3). Some studies have reported an association between mitochondrial dysfunction and NASH [30, 31]. The present results also indicate that the increased MPO activity in Kupffer cells and the liver mitochondrial dysfunction induced the oxidative damage in NASH. Whereas rats supplied VCPL showed an increase in SOD-like activity in plasma (Fig. 2), and showed a decrease of MPO activity and mitochondrial ROS derivation in the liver.

It has been reported that the SOD activities in mice and rats are increased by the oral administration of the fruit, peel and seeds of grapes [32, 33], and by VCPL in our previous study to examine the preventive effect on NASH [12]. Though it is unclear what substances elevate the plasma SOD-like activity by VCPL administration, we expect that the elevation reflects the antioxidative constituents absorbed from the gastrointestinal tract into circulation or, through them, the preservation of endogenous antioxidants. Therefore, further studies are necessary to identify this ingredient in VCPL. Nevertheless, the present results have shown that VCPL administration could help to slow the progress of NASH by scavenging ROS, by correcting the excess generation of ROS, or by both these actions.

Inflammation is thought to also be a key element in the progression of NASH, and intimately related to OS. Activation of NF-κB is particularly critical for the development of inflammation under a variety of conditions [34]. NF-κB is also reported to be an OS-responsive transcription factor [35–37]. In fact, OS is invariably recognized in the liver of patients with NASH [37–39] and in several rodent models of steatohepatitis [40, 41]. The present study showed that the NF-κB expression in the nuclear fraction of hepatocytes increased (Fig. 5) in correspondence with

the elevation of mitochondrial ROS derivation in NASH rats. And these levels were decreased by VCPL administration respectively. Thus we suggest that VCPL inhibited activation of NF-κB by regulating OS, leading to slow the progress of NASH.

This study is the first to demonstrate that VCPL can ameliorate NASH in the progressive stage by increasing plasma antioxidative activity, decreasing the ROS derivation from mitochondrial energy metabolism and reducing the NF-κB activation in a rat model, suggesting that OS-induced damage and inflammation are associated with the progressive NASH. Further studies are required to determine the mechanisms underlying the beneficial effect of the antifibrotic activity of VCPL on progressive NASH, as well as to assess the serial changes in mitochondrial function and liver architecture in the NASH livers.

In conclusion, this study demonstrated that the antioxidative activity of VCPL seems to improve progressive NASH by breaking the crosstalk between OS and inflammation, suggesting that VCPL is a novel functional food to slow the progress of NASH.

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