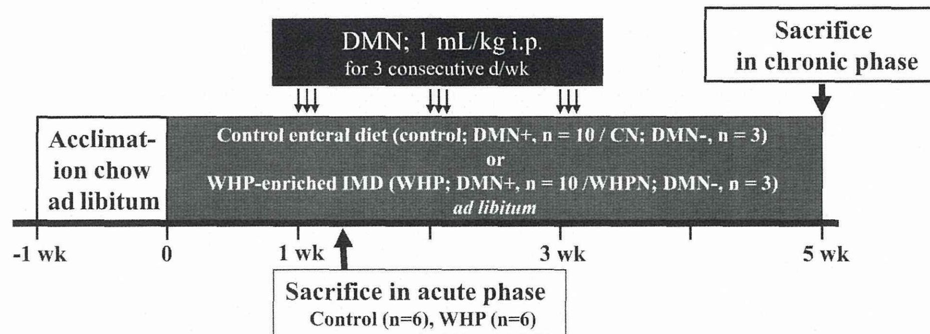


**Fig. 1.** Experimental protocols in vivo. CN, control diet without DMN as negative control for DMN; DMN, dimethylnitrosamine; IMD, immunomodulating diet; WHP, whey-hydrolyzed peptide; WHPN, whey-hydrolyzed peptide diet without DMN as negative control for DMN.

production extracellular matrix and leads to liver fibrosis [7,8]. Reactive oxygen species (ROS) are involved in liver injury, and antioxidants may protect hepatocytes and prevent following liver fibrosis [9]. Moreover, hepatocellular damages and inflammatory cytokines interact with each other.

Recent nutritional studies demonstrated that bovine milk proteins have many effects for health benefits, such as preventing weight gain and enhancing bone architecture [10]. Whey-hydrolyzed peptide (WHP) is a major peptide component of bovine milk. A WHP-enriched diet is one form of immunomodulating diet (IMD) that has been widely used for nutritional support in patients with malnutrition or insufficient oral intake. A WHP-enriched IMD exerts antioxidant, anti-inflammatory, immunomodulating, and antibacterial effects in some experimental models [11–15]. The clinical utility of this novel WHP-enriched IMD has been documented, mainly at the perioperative period [16–18]. Previous research has indicated that a WHP-enriched diet exerts an anti-inflammatory effect, and may have therapeutic potential [11].

The present study investigated the antifibrotic effects of WHP in a rat liver cirrhosis model with progressive fibrosis. We also discuss the therapeutic potential of WHP against progressive fibrosis and possible mechanisms during liver cirrhosis progression from a literature review.

## Materials and methods

### *Rats*

Male Sprague-Dawley rats (6 wk old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Rats were cared for according to the Institutional Guidelines for Animal Welfare. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Kyoto University (Protocol ID: MedKyo12521).

### *Immunomodulating diet*

Rats were fed conventional chow as a control enteral diet (MEIBALANCE; Meiji Dairies Co., Tokyo, Japan) or a WHP-enriched IMD (MEIN; Meiji Dairies Co.) ad libitum throughout the experiments (Fig. 1). The compositions of the two diets were almost identical, except for the protein source as summarized in Table 1 (casein in the control diet, and WHP in the WHP-enriched IMD).

### *Liver cirrhosis model rats*

One week after starting diets, rats were intraperitoneally given dimethylnitrosamine (DMN; 1% dissolved in saline; 1 mL/kg; Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) consecutively for 3 d each week for 3 wk to induce progressive fibrosis and subsequent cirrhosis [19,20] (Fig. 1).

### *Histopathologic assessment*

Liver specimens were fixed in 4% paraformaldehyde, embedded in paraffin, serially cut into thin slices (3 μm thick). Slides were stained with hematoxylin

**Table 1**
Diet composition*

| | Control | WHP-enriched IMD |
|---|---|---|
| Proteins (g) | 5.0 | 5.0 |
| Protein (% kcal) | 20.0 | 20.0 |
| Protein sources | Total milk protein<br>Na caseinate | Whey peptides<br>Fermented milk |
| Carbohydrates (g) | 15.3 | 14.5 |
| Carbohydrates (% kcal) | 57.5 | 55 |
| CHO sources | Dextrin | Isomaltulose<br>Dextrin |
| Lipids (g) | 2.50 | 2.80 |
| Lipids (% kcal) | 22.5 | 25.0 |
| MCT (g) | - | 0.59 |
| EPA, DHA (g) | - | 0.060 |
| ω-6/ω-3 | 3.2 | 2.0 |
| Vitamins | | |
| Vitamin A (g RE) | 60.0 | 150 |
| Vitamin D (g) | 0.50 | 0.75 |
| Vitamin E (mg) | 3.0 | 5.0 |
| Vitamin K (g) | 5.0 | 3.4 |
| Vitamin B1 (mg) | 0.15 | 0.25 |
| Vitamin B2 (mg) | 0.20 | 0.30 |
| Niacin (mg) | 1.6 | 3.0 |
| Vitamin B6 (mg) | 0.30 | 0.30 |
| Vitamin B12 (g) | 0.60 | 0.60 |
| Folic acid (g) | 50 | 50 |
| Biotin (g) | 15 | 7.5 |
| Pantothenic acid | 0.60 | 1.2 |
| Vitamin C (mg) | 16 | 50 |
| Choline (mg) | 1.8 | 9.2 |
| Carnitine (mg) | - | 15 |
| Minerals | | |
| Sodium (mg) | 110 | 70 |
| Potassium (mg) | 100 | 80 |
| Calcium (mg) | 70 | 80 |
| Magnesium (mg) | 30 | 20 |
| Phosphorus (mg) | 70 | 70 |
| Iron (mg) | 1.0 | 1.0 |
| Zinc (mg) | 1.0 | 1.0 |
| Copper (mg) | 0.050 | 0.050 |
| Manganese (mg) | 0.230 | 0.175 |
| Chromium (g) | 3.00 | 2.96 |
| Molybdenum (g) | 2.5 | 2.5 |
| Selenium (g) | 6.0 | 5.0 |
| Iodine (g) | 15 | 9.7 |
| Chloride (mg) | 110 | 80 |

CHO, carbohydrate; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IMD, immunomodulating diet; RE, retinol equivalent; MCT, medium chain triglyceride; WHP, whey-hydrolyzed peptide; (-), no additives

* The major difference between WHP-enriched IMD and control enteral diet used in this study is the protein composition; the WHP-enriched IMD contains whey peptides and fermented milk product as the protein sources, whereas the control enteral diet contains total milk protein and sodium caseinate.

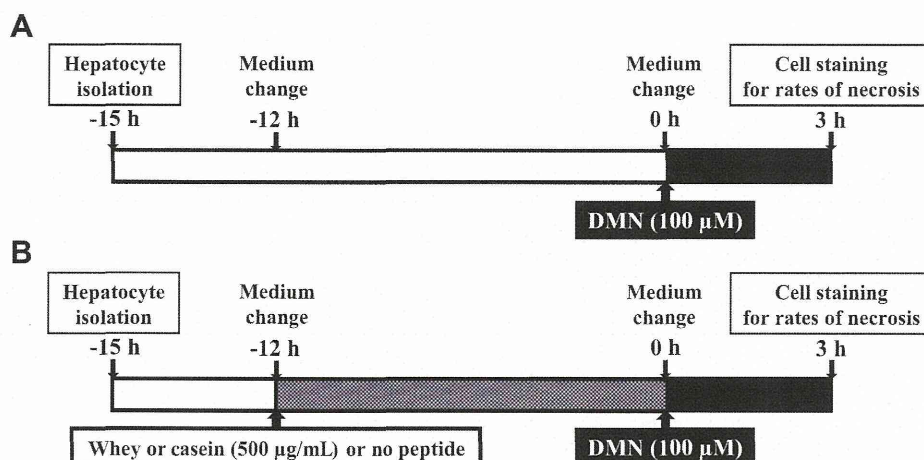


**Fig. 2.** Protocol for analysis of the hepatocyte-protective effect. (A) Protocol for analysis of the hepatocyte-protective effect in vivo for isolated hepatocytes from rats fed a WHP diet. (B) Protocol for analysis of the hepatocyte-protective effect in vitro for hepatocytes with WHP-added medium. DMN, dimethylnitrosamine; WHP, whey-hydrolyzed peptide.

and eosin and Mallory–Azan stain for histologic analysis of fibrosis. The degrees of liver fibrosis and histologic activity in native livers were scored according to the Metavir scoring system [21].

*Immunohistochemistry*

After blocking, sections were incubated with a primary antibody for α-smooth muscle actin (α-SMA) (ab5694; Abcam, Cambridge, UK) and then with a labeled polymer (Envision System/Horse radish peroxidase; Dako, Tokyo, Japan). Sections were examined after incubation with diamino benzidine (Liquid Diamino Benzidine Substrate Chromogen System; Dako). The α-SMA staining was analyzed using quantitative software (BZ-Analyzer; KEYENCE, Osaka, Japan), with detection thresholds set to the brown color (diamino benzidine color). Images of five non-overlapping fields were selected at random and captured per section at ×200 magnification. The degree of labeling in each section was determined from the area within the color range divided by the total area. For collagen I, we used primary antibody against collagen I (NB600-408; Novus Biologicals, Littleton, CO, USA) with biotinylated antibody-recognizing rabbit immunoglobulin G (BA-1000; Vector Laboratories, Burlingame, CA, USA). The positive areas of the staining were analyzed by quantitative software in the same way as α-SMA staining.

*Hydroxyproline determination*

For collagen quantification in the liver, hydroxyproline (the specific amino acid of type I collagen) was measured using the standard biochemical method described previously [22] Hydroxyproline was then quantitated photometrically at 558 nm from a standard curve generated using purified hydroxyproline (Sigma, Tokyo, Japan).

*Hepatocyte isolation*

Hepatocytes were isolated from rat livers as previously described [23,24]. Briefly, rat liver was perfused for 15 min with 0.03% collagenase (Wako, Kyoto, Japan). After collagenase perfusion, the cell suspension was centrifuged at 50g for 1 min.

*Cell culture*

Isolated hepatocytes were cultured on six-well plates coated with type I collagen at a cell density of $1 \times 10^5$ cells/well with serum-free Medium 199 Earle's liquid (Invitrogen, Tokyo, Japan), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL of amphotericin B at 37°C.

*Propidium iodide Hoechst ratio calculation for degree of hepatocyte necrosis*

Cultured hepatocytes were stained with Hoechst 33342 (Nacalai Tesque, Kyoto, Japan) and propidium iodide (Calbiochem, San Diego, CA, USA) for DNA staining to evaluate cell viability. Double staining with propidium iodide (for dead cells) and Hoechst 33342 (for dead and viable cells) was used to differentiate necrotic cells from normal cells with a fluorescent microscope. Images of five non-overlapping fields at ×200 magnification were selected at random, and cells were automatically counted (BZ-II Analyzer; KEYENCE). The propidium iodide-positive ratio was calculated as the percentage of propidium iodide-positive cells among Hoechst 33342-positive cells [24].

*Inflammatory cytokines in serum*

Serum concentrations of IL-6, TNF-α, IL-10, and interferon (IFN)-γ were measured using conventional assay kits (Cytometric Bead Array Kits; Becton Dickinson Co., Franklin Lakes, NJ, USA).

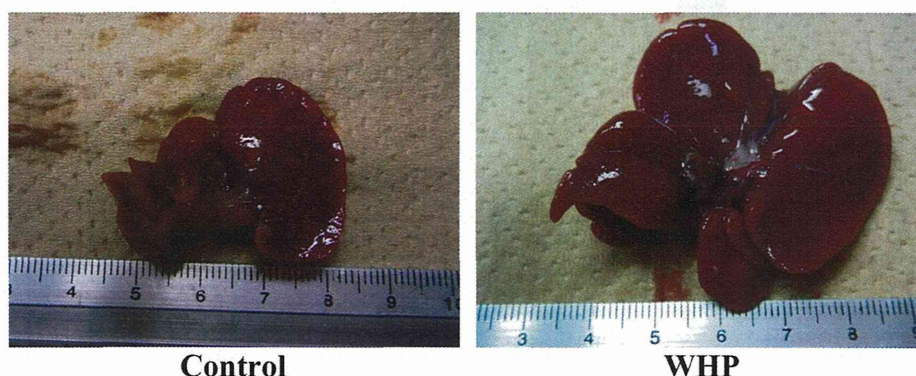


**Fig. 3.** Antifibrotic/cirrhotic effects of WHP in macroscopic findings of livers in the DMN-induced cirrhosis rat model (chronic phase). In the control group, a granular liver surface and remarkable atrophy were observed. Livers from the WHP group showed near normal findings and a non-damaged appearance. DMN, dimethylnitrosamine; WHP, whey-hydrolyzed peptide.

**Table 2**
Degree of cirrhosis (chronic phase)*

| | Control (n = 10) | WHP (n = 10) | *P*-values |
|---|---|---|---|
| LC | 6 | 0 | 0.003 |
| Ascites | 6 | 0 | 0.003 |
| Body weight (g) | 302 ± 27 | 307 ± 26 | 0.650 |
| Liver weight (g) | 6.8 ± 2.5 | 10.5 ± 1.9 | 0.007 |
| Spleen weight (g) | 1.7 ± 0.3 | 0.8 ± 0.2 | <0.001 |

DMN, dimethylnitrosamine, LC, liver cirrhosis; WHP, whey-hydrolyzed peptide
* Macroscopic LC, ascites, liver atrophy, and splenomegaly were significantly suppressed in the WHP group in a DMN-induced cirrhosis rat model.

*Quantitative reverse-transcription polymerase chain reaction analysis*

Total RNA was extracted from liver samples using an assay kit (RNeasy Mini Kit with on-column DNA digestion; Qiagen, Tokyo, Japan). Total RNA was reverse transcribed to complementary DNA using an assay kit (Omniscript RT Kit; Qiagen, Valencia, CA, USA). Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) for IL-6 and TNF-$\alpha$ was performed (TaqMan Fast Universal PCR Master Mix; Applied Biosystems, Foster City, CA, USA) using a real-time quantitative thermal cycler (StepOnePlus; Applied Biosystems). Validated exon-spanning primers (TaqMan primers [B-actin Rn 00667869 _m1; IL-6 Rn 01410330_m1; TNF-$\alpha$ Rn 00562055_m1]; Applied Biosystems) were used during amplification with B-actin as the internal control. PCR products were quantified using the 2-$\Delta\Delta$CT method [25]. Quantitative RT-PCR for cytochrome P450 (CYP) 2 E1 was performed (SYBR Green Master reaction mix; Invitrogen, Carlsbad, CA, USA) using a real-time quantitative thermal cycler (StepOnePlus; Applied Biosystems). The relative abundance of the target genes was obtained by calculating against a standard curve and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control. Primer sequences used were as follow:

CYP2 E1-Forward: 5′-AGTCTGCCACCCTCTGCTTA-3′, CYP2 E1-Reverse: 5′-GAAAGCTGAGACCCATGAGC-3′, GAPDH-Forward: 5′-TGGAGTCTACTGGCGTCTT-3′, GAPDH-Reverse: 5′-TGTCATATTTCTCGTGGTTCA-3′.

*Reactive oxygen species analysis for liver specimens in acute and chronic phases*

Malondialdehyde (MDA) levels, a quantitative value of ROS, were determined according to the thiobarbituric acid method using a conventional assay kit (NWLSS MDA Assay Kit; Northwest Life Science Specialties, Vancouver, WA, USA). Glutathione (GSH) levels were determined using a GSH quantification assay kit (GSSG/GSH Quantification Kit; Dojindo, Tokyo, Japan). Biochemical measurements were carried out at room temperature using a spectrophotometer (Molecular Devices, Tokyo, Japan).

*Study design*

*Liver cirrhosis model of rat fed WHP with DMN: Chronic phase*

The control group (control diet with DMN treatment; n = 10) and the CN group (control diet without DMN as negative control for DMN; n = 3) received the control enteral diet, and the WHP group (WHP diet with DMN; n = 10) and WHPN group (WHP diet without DMN as negative control for DMN; n = 3)

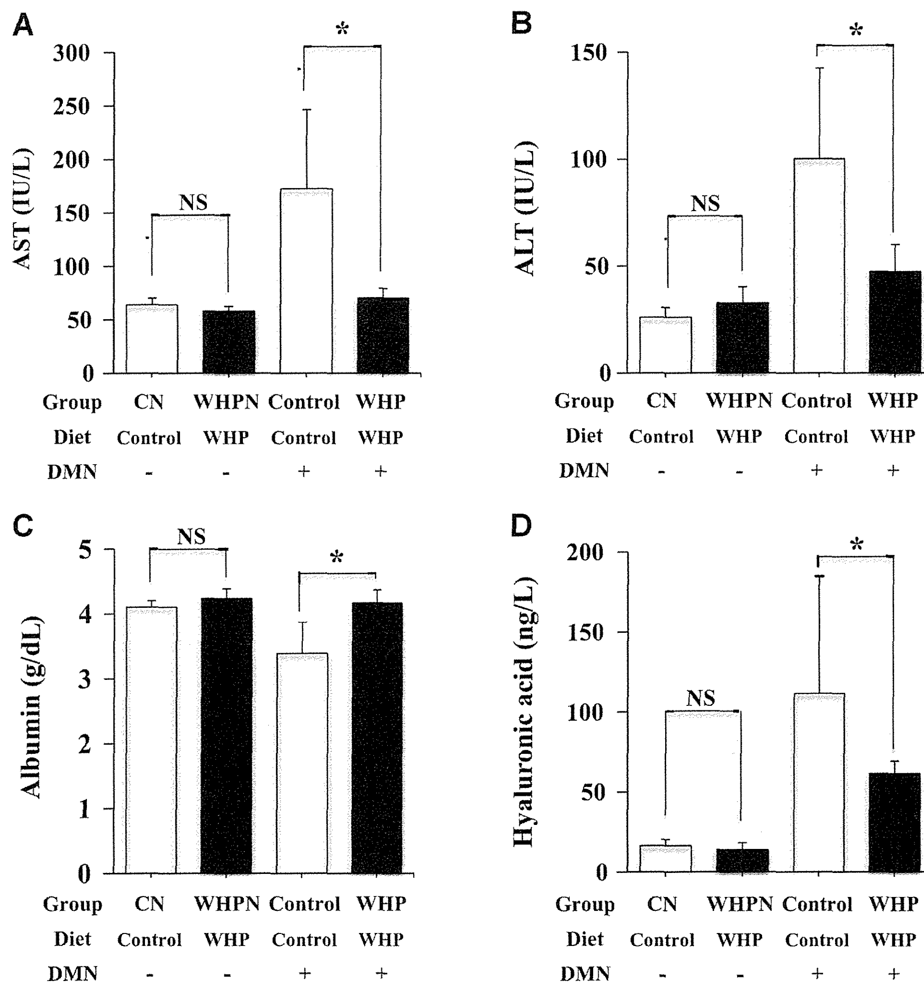


**Fig. 4.** Antifibrotic/cirrhotic effects of WHP in serum biochemical examinations (chronic phase). Increased serum levels of liver enzymes (A, B) and hyaluronic acid (D) were markedly attenuated, and albumin (C) was significantly higher in the WHP group than in the control group. *$P < 0.05$; NS, not significant ($P \geq 0.05$). CN, control diet without DMN as negative control for DMN; DMN, dimethylnitrosamine; WHP, whey-hydrolyzed peptide; WHPN, whey-hydrolyzed peptide diet without DMN as negative control for DMN.

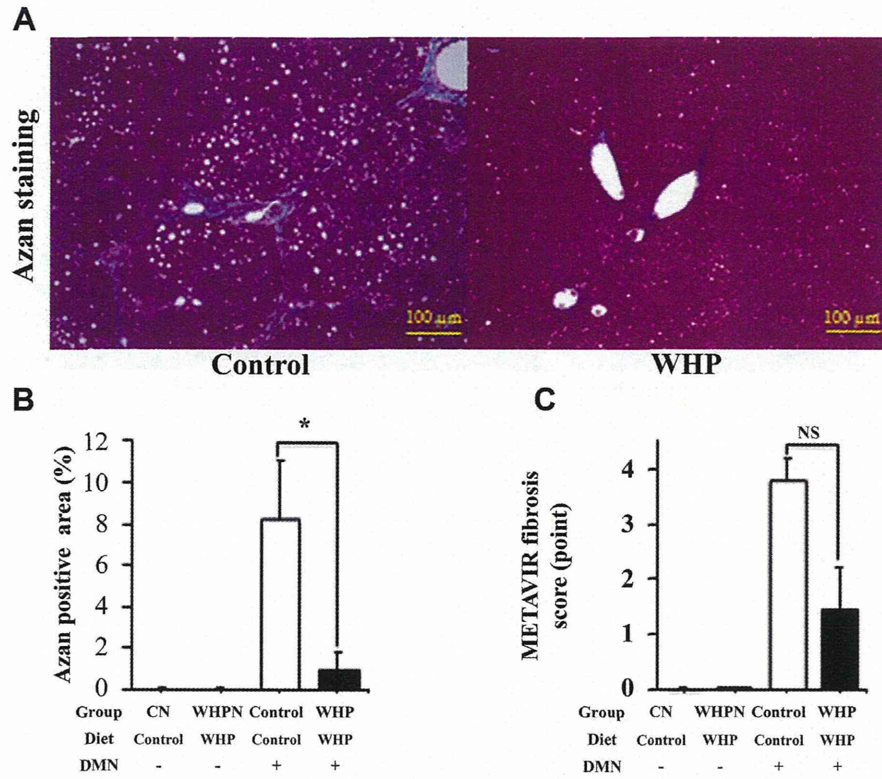


**Fig. 5.** Antifibrotic/cirrhotic effects of WHP in Azan staining (A, B) and Metavir fibrosis scores (C) (chronic phase). Azan-positive areas (A) and Metavir fibrosis scores (B) were significantly lower in the WHP group than in the control group. $^*P < 0.05$. CN, control diet without DMN as negative control for DMN; DMN, dimethylnitrosamine; WHP, whey-hydrolyzed peptide; WHPN, whey-hydrolyzed peptide diet without DMN as negative control for DMN.

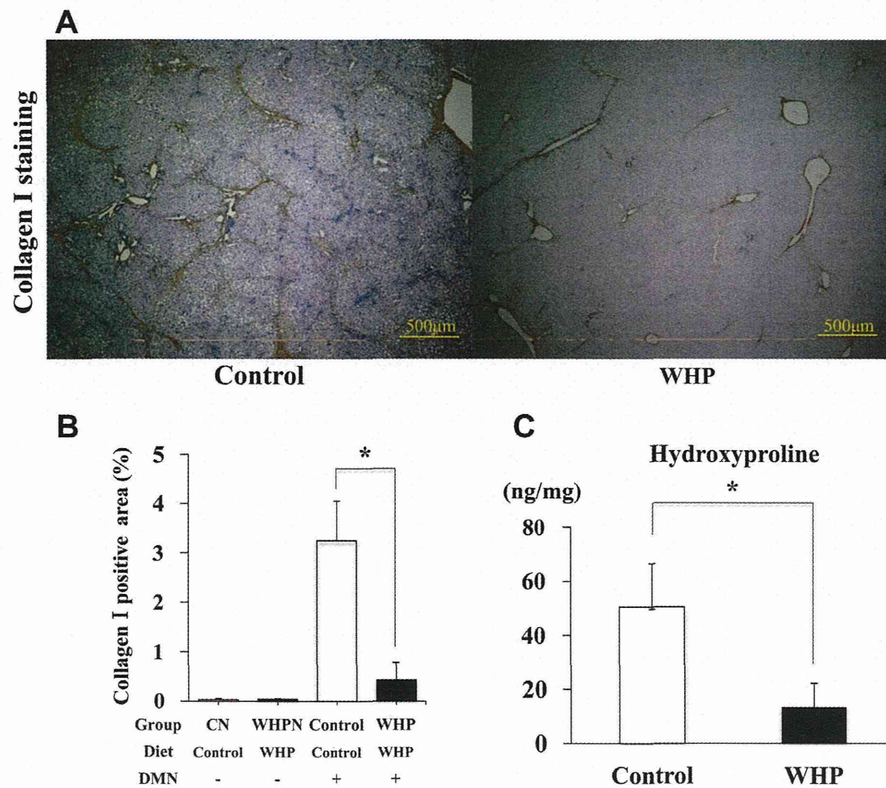


**Fig. 6.** Reduced rates of collagen expression in collagen I staining and hydroxyproline content in liver tissue by WHP treatment. Collagen I positive area (A, B) and hydroxyproline content in liver tissue (C) were significantly fewer in the WHP group than in the control group. $^*P < 0.05$. CN, control diet without DMN as negative control for DMN; DMN, dimethylnitrosamine; WHP, whey-hydrolyzed peptide; WHPN, whey-hydrolyzed peptide diet without DMN as negative control for DMN.

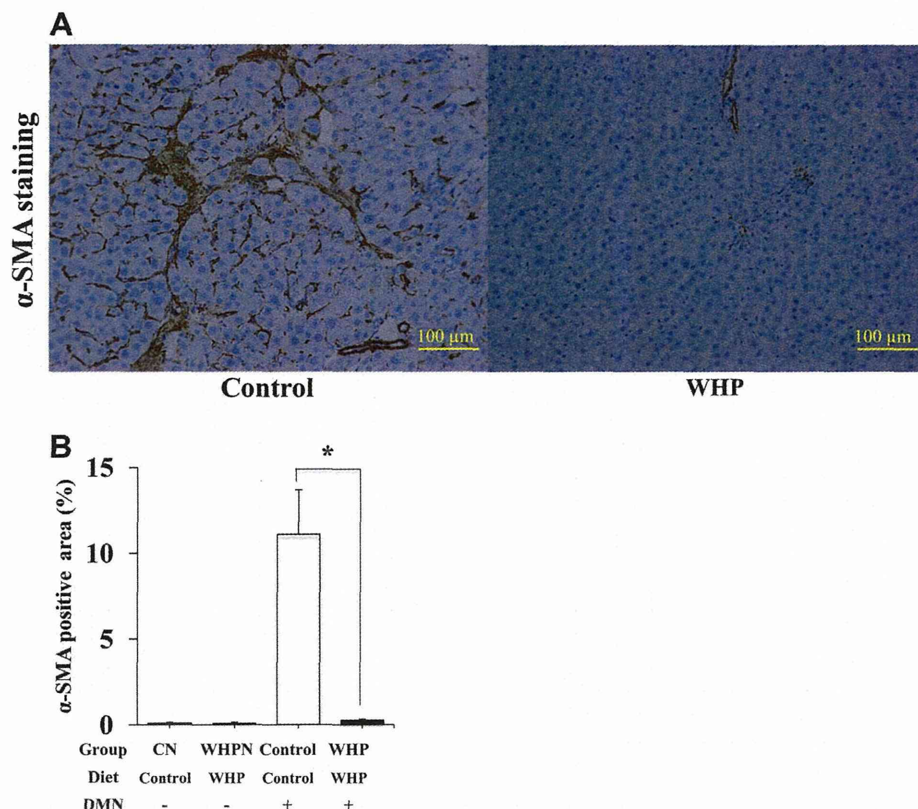


**Fig. 7.** Prevention of activation of hepatic stellate cells in α-SMA staining by WHP. The number of α-SMA-positive areas, which reflected to activated hepatic stellate cells, were significantly fewer in the WHP group than in the control group (A, B). *$P < 0.05$. DMN, dimethylnitrosamine; SMA, smooth muscle actin; WHP, whey-hydrolyzed peptide; WHPN, whey-hydrolyzed peptide diet without DMN as negative control for DMN.

received the WHP-enriched IMD ad libitum. Twelve d after the last DMN treatment, rats were sacrificed under isoflurane anesthesia (Escain; Mylan, Tokyo, Japan) (Fig. 1). At sacrifice, blood samples were collected from the abdominal aorta for conventional liver function tests (aspartate aminotransferase [AST], alanine aminotransferase [ALT], and total bilirubin [T-Bil]). Livers and spleens were excised and weighed for histopathology and immunochemistry.

To confirm the anti-inflammatory effect of WHP as previously reported, serum levels of IL-6, TNF-α, IL-10, IFN-γ and TGF-β, and also mRNA levels of IL-6 and TNF-α in liver tissue were measured. CYP2 E1 mRNA expression was measured to examine whether CYP2 E1 or a metabolic enzyme of DMN was regulated by WHP treatment. Also, MDA and GSH in liver tissue were measured to clarify whether WHP treatment was related to ROS reduction.

*Liver cirrhosis model of rat fed WHP with DMN: Acute phase*

The acute phase was also investigated before reaching the chronic phase. The control group (n = 6) received a control enteral diet, and the WHP group (n = 6) received a WHP-enriched IMD ad libitum. One d after the last DMN injection for 1 wk, rats were sacrificed (Fig. 1). At sacrifice, blood samples and liver tissues were collected. Serum levels of inflammatory cytokines were examined as described previously. For liver tissue, expression of mRNA of inflammatory cytokines and CYP2 E1, MDA, and GSH also were measured as described previously.

We performed additional experiments to evaluate the synergistic effects of TNF-α inhibitor (pentoxyfilline) in acute phase (pentoxifylline [PTX]-combined model). Rats were given DMN intraperitoneally and PTX [26–28] concomitantly for 3 consecutive days and sacrificed 24 h after last treatment in four experimental groups (n = 6 for each group, all groups were treated with DMN): control diet without PTX (ACDN), control diet with PTX (ACDP), WHP diet without PTX (AWDN), and WHP diet with PTX (AWDP). Serum conventional liver function tests, serum cytokines and histopathologic data were measured.

*Hepatocyte-protective effect in vivo for isolated hepatocytes from rats fed WHP diet*

Before hepatocyte isolation, rats were fed a control enteral diet (control diet group) or a WHP-enriched IMD (WHP diet group) for 1 wk. Hepatocytes were isolated from rat livers by a perfusion solution containing collagenase. Isolated hepatocytes were cultured on six-well plates. Medium was replaced 3 h after plating. Twelve hours later, 100 μmol/L of DMN was added [23], and cells were incubated. Three hours after DMN challenge, cultured hepatocytes were stained with Hoechst 33342 and propidium iodide. The propidium iodide-positive ratio was calculated to evaluate the degree of hepatocyte necrosis (Fig. 2A) [24].

*Hepatocyte-protective effect in vitro for hepatocytes with WHP-added medium*

Hepatocytes from rats fed commercial chow for 1 wk were isolated and cultured as above. Three hours after plating, medium was replaced with serum-free Medium 199 Earle's liquid without or with 500 μg/mL of casein peptide hydrolyzed by trypsin (CHP; Meiji Dairies Co.) or with 500 μg/mL of WHP (WHP by trypsin; Meiji Dairies Co.). Twelve hours later, 100 μM DMN or medium alone was added, and cells were incubated. Six experimental groups were defined as follows: Medium alone without peptide (D–*P*– group; n = 6), CHP alone (D–C+ group; n = 6), WHP alone (D–W+ group; n = 6), DMN alone (D+*P*– group; n = 6), DMN with CHP (D+C+ group; n = 6), and DMN with WHP (D+W+ group; n = 6). Three hours after DMN challenge, cultured hepatocytes were stained with Hoechst 33342 and propidium iodide. The propidium iodide-positive ratio was calculated as previously described (Fig. 2B).

*Statistical analyses*

Continuous values are expressed as means ± SD, and shown as the control group first and then the treated group. Data were statistically analyzed using Prism 5.0.1 (SAS Institute, Cary, NC, USA). Discrete variables were compared using a $\chi^2$ test. Continuous variables were analyzed using a

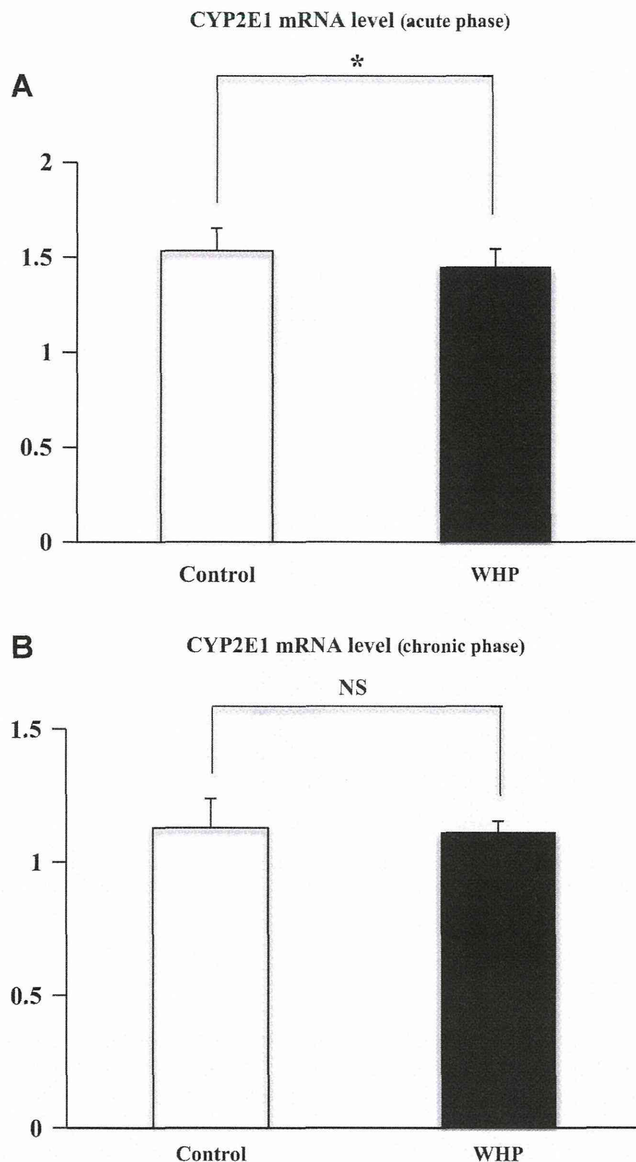


**Fig. 8.** Down-regulation of CYP2 E1 and restrained production of toxic metabolites by WHP treatment. In the chronic phase, CYP2 E1 mRNA levels showed no significant difference between the two groups (B). In the acute phase, CYP2 E1 mRNA levels were statistically significantly lower in the WHP group than in the control group (A). $^*P < 0.05$; NS, not significant ($P \geq 0.05$). CYP, cytochrome P450; WHP, whey-hydrolyzed peptide.

Student's $t$ test or two-way or one-way analysis of variance followed by a Bonferroni post hoc comparison test. $P$-value $< 0.05$ was considered statistically significant.

## Results

*Liver cirrhosis model of rat fed WHP with DMN: Chronic phase*

Six of 10 rats in the control group showed a macroscopic granular liver surface, liver atrophy, splenomegaly, and ascites. All rats in the WHP group clearly showed near-normal liver appearance and no ascites (Fig. 3, Table 2). Body weights of rats at sacrifice did not differ between the groups ($P = 0.650$) (Table 2). Mean liver wet weights were significantly lower in control group than in the WHP group ($P = 0.007$) (Table 2). Mean spleen wet weights were significantly higher in control group than in the WHP group ($P < 0.0001$) (Table 2).

Increased liver enzyme and hyaluronic acid serum levels were also markedly attenuated in the WHP group compared with the control group (control versus WHP: AST, 165.1 ± 71.2 versus 70.1 ± 9.1 IU/L, $P < 0.001$; ALT, 100.2 ± 42.3 versus 47.1 ± 13.3 IU/L, $P < 0.001$; hyaluronic acid, 291.2 ± 330.3 versus 21.8 ± 7.8 ng/mL, $P = 0.009$; Fig. 4A, B, and D). Serum albumin was significantly higher in the WHP group than in the control group (control versus WHP: 3.39 ± 0.48 versus 4.21 ± 0.14 g/dL, $P < 0.001$; Fig. 4C).

With Mallory-Azan (Fig. 5A) and collagen type I (Fig. 6A) stainings, radiating fibrous septa were observed in the control group but not in the WHP group. The percentage of Azan-positive areas (Fig. 5B) and Metavir fibrosis scores (Fig. 5C) were significantly fewer in WHP group than those in control group (control versus WHP: Azan-positive areas, 8.26% ± 2.87% versus 1.08% ± 0.77%, $P < 0.001$; Metavir fibrosis scores, 3.8 ± 0.4 versus 1.5 ± 0.7 points, $P < 0.001$). Percent of collagen type I-positive area (Fig. 6B) and hydroxyproline content in liver tissue (Fig. 6C) were markedly lower in the WHP group compared with the control group (control versus WHP: collagen type I-positive areas, 3.25% ± 0.80% versus 0.43% ± 0.36%, $P = 0.002$; hydroxyproline content: 505.0 ± 159.2 versus 134.8 ± 88.2 ng/mg, $P = 0.002$). The α-SMA staining showed the presence of activated hepatic stellate cells in the control group but not in the WHP group (Fig. 7A). The percentage of α-SMA-positive areas in the WHP group were significantly fewer than in the control group (control versus WHP: 11.09% ± 2.61% versus 0.25% ± 0.07%, $P < 0.001$) (Fig. 7 B). The WHP diet group without DMN treatment (WHPN) did not show any liver damage and data, and there were also no significant differences between the CN and WHPN groups in macroscopic appearance, liver and spleen weight, serum liver function tests (Fig. 4), and microscopic data (Fig. 5A–C, 6A and B, and 7A and B).

In the chronic phase, serum levels of IL-6, TNF-α and TGF-β were too low to be detected (under detection limits). CYP2 E1 mRNA levels revealed no significant differences between groups (Fig. 8B). MDA levels (a quantitative value of ROS) were significantly higher in the control group (17.89 ± 4.25 μmol/L/g) than those in the WHP group (4.84 ± 1.49 μmol/L/g, $P < 0.001$; Fig. 9B). GSH levels were significantly higher in the WHP group (6.21 ± 1.92 μmol/L/g) than in the control group (2.86 ± 1.12 μmol/L/g, $P = 0.004$; Fig. 9D).

*Liver cirrhosis model of rat fed WHP with DMN: Acute phase*

In the acute phase, elevated serum levels of liver enzymes (AST and T-Bil) were significantly attenuated in the WHP group compared with the control group (control versus WHP: AST, 1111 ± 385.7 IU/L versus 617 ± 19.8 IU/L, $P = 0.023$; T-Bil, 0.42 ± 0.13 versus 0.27 ± 0.09 mg/dL, $P = 0.003$; Fig. 10A, B). IL-10 (Fig. 11A), TNF-α (Fig. 11B), and TGF-β (Fig. 11E) serum levels were significantly lower in the WHP group than in the control group (control versus WHP: IL-10, 160.8 ± 40.1 versus 92.4 ± 51.0 pg/mL, $P = 0.026$; TNF-α, 140.0 ± 22.2 versus 101.1 ± 51.0 pg/mL, $P = 0.049$; TGF-β, 582.2 ± 166.2 versus 390.9 ± 43.1 pg/mL, $P = 0.029$). IL-6 serum levels did not show significant differences between groups (Fig. 11C). IL-6 mRNA levels in liver tissue also showed no remarkable differences between groups (Fig. 11D). CYP2 E1 mRNA levels were significantly lower in the WHP group than in the control group (control versus WHP: 1.53 ± 0.02 versus 1.45 ± 0.09, $P = 0.024$) in the acute phase (Fig. 8A). MDA levels in the acute phase were significantly lower in the WHP group than in the control group (control versus WHP, 20.3 ± 3.0 versus 11.0 ±

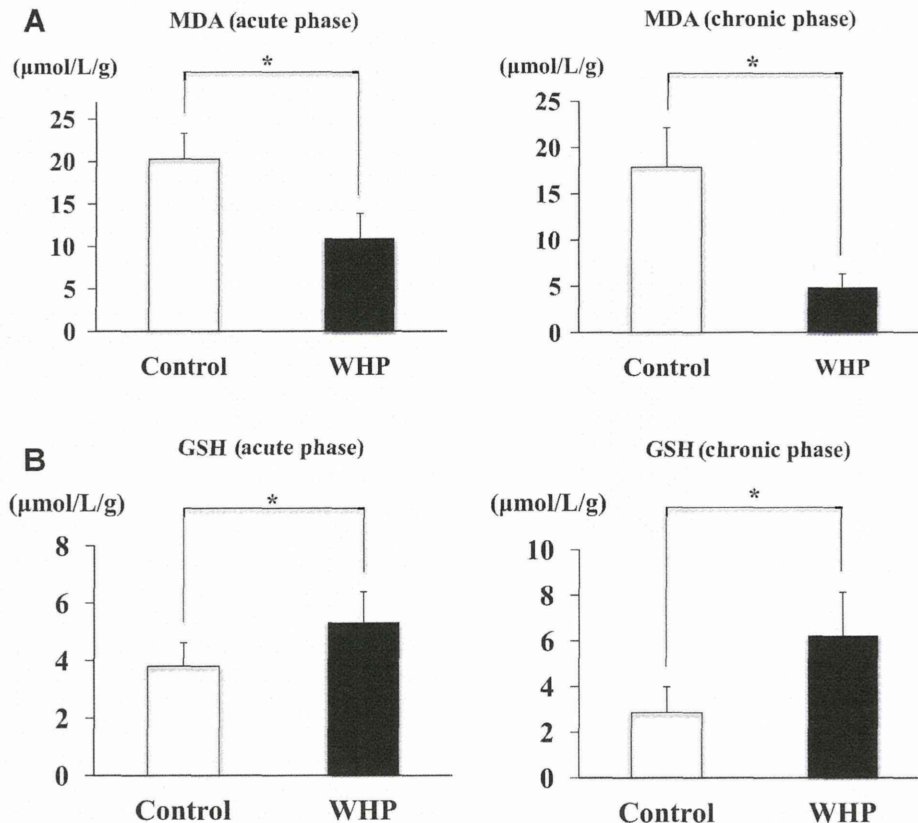


**Fig. 9.** Antioxidant effect of WHP to reduce ROS and keep GSH higher in liver. MDA levels, a quantitative variable of ROS, were significantly lower in the WHP group than in the control group in acute and chronic phase (A). GSH levels were significantly higher in the WHP group than those in control group (B). $^{*}P < 0.05$. GSH, glutathione; MDA, malondialdehyde; ROS, reactive oxygen species; WHP, whey-hydrolyzed peptide.

3.0 μmol/L/g, $P = 0.006$; Fig. 8A). GSH levels were significantly higher in the WHP group than in the control group (control versus WHP, 3.79 ± 0.82 versus 5.31 ± 1.07 μmol/L/g, $P = 0.014$; Fig. 9A). In a PTX-combined model, there were no significant differences between AWDN and AWDP in all liver damage data (serum AST, ALT, T-Bil, hyaluronic acid, necrotic area and Metavir histologic activity scores). There were also no significant differences in liver damage data between ACDN and ACDP, except in serum ALT ($P < 0.05$). On the other hand, there were significant differences between ACDN and ACDP (data in the ACDP group were lower than in the ACDN group) in TNF-α and IL-6 ($P < 0.05$, respectively), between AWDN and AWDP (data in the AWDP group were lower than in the AWDN group) in TNF-α ($P < 0.05$; Fig. 12).

*Hepatocyte-protective effect in vivo for isolated hepatocytes from rats fed WHP diet*

The propidium iodide-positive ratios of cultured hepatocytes were significantly lower in the WHP group compared with the control group (control versus WHP: 28.6% ± 8.9% versus 9.9% ± 3.6%, $P < 0.001$; Fig. 13A, B).

*Hepatocyte-protective effect in vitro for hepatocytes with WHP-added medium*

The propidium iodide-positive ratios were markedly lower in D+W+ group than that in D+P– group (control versus WHP: 41.5% ± 14.0% versus 5.8% ± 1.6%, $P < 0.001$; Fig. 13C).

## Discussion

WHP is a major peptide component of natural bovine milk. A WHP-enriched diet is one form of IMD. A WHP-enriched IMD exerts anti-inflammatory, immunomodulating, and antibacterial effects mainly in acute liver injury models [11–15]. It has been demonstrated that a WHP-enriched diet suppresses D-galactosamine or lipopolysaccharide-induced hepatitis [13]. As previously shown, a WHP-enriched diet protects against carbon tetrachloride-induced hepatitis [15]. Such hepatotoxins induce the production of inflammatory cytokines. The studies just cited reported that the plasma levels of cytokines (such as TNF-α, IL-1β, and IL-6) increased in rodents fed a control diet and clearly decreased in rodents fed a WHP-enriched diet [13–15]. These findings suggest that WHP inhibited the meditation of inflammation by cytokine production. The clinical utility of this novel WHP-enriched IMD has been mainly documented at the perioperative period [16–18]. Previous research has indicated that a WHP-enriched diet exerts an anti-inflammatory effect and may have therapeutic potential [11].

The present study demonstrated that a WHP-enriched IMD exerted an antifibrotic effect and prevented subsequent cirrhosis in rats that had been subject to repeated DMN injections. WHP clearly prevented the development of a macroscopic granular liver surface, liver atrophy, splenomegaly, and ascites; characteristics that were observed in rats fed without WHP added to their diet (Fig. 3A, Table 2). Histopathologic fibrosis and increased levels of serum hyaluronic acid, which are accurate

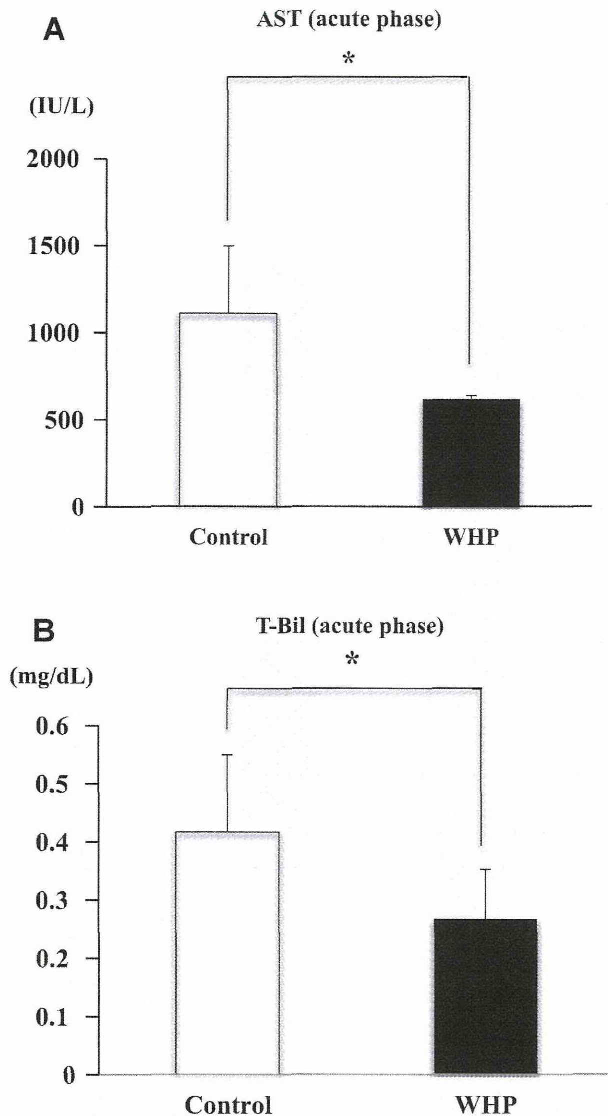


**Fig. 10.** Conventional liver function tests in the acute phase. AST and T-Bil levels were significantly attenuated in the WHP group. $*P < 0.05$. AST, aspartate aminotransferase; T-Bil, total bilirubin; WHP, whey-hydrolyzed peptide.

markers of fibrosis in the clinical setting, were markedly attenuated in the WHP group compared with the control group (Figs. 3–6). The findings indicated that oral intake of a WHP-enriched diet for ~5 wk almost completely prevented liver fibrosis and subsequent cirrhosis induced by DMN. The group of rats fed a WHP diet for 5 wk without DMN did not show any liver damage data. These data suggested that a WHP diet is safe and suitable for long-term feeding. To the best of our knowledge, this is the first study to report that WHP has an antifibrotic effect and prevents progression to cirrhosis in a chronic inflammatory model.

To elucidate the mechanisms of the antifibrotic effect of WHP, we focused inflammatory reactions via cytokines. Many reports have previously described the anti-inflammatory effects of WHP in IMD [11–15,19,29,30]. In the chronic phase in vivo, plasma levels of inflammatory cytokines (TNF-α, IL-1β, IL-6 and TGF-β) were very low in both groups (data not shown). A possible explanation for this observation is that inflammatory cytokine levels were already falling at the point of our time-point sampling after 2 wk from last DMN administration. In the acute phase, elevated liver enzyme and T-Bil serum enzymes were significantly attenuated in the WHP group. IL-10 TNF-α and TGF-β1 levels in serum were significantly lower in the WHP group than those in the control group. The data of liver test and IL-10 and TNF-α (but not IL-6) correspond with the current explanation for the anti-inflammatory effect of WHP. IL-6 may not play a key role in the anti-inflammatory effects of WHP in the process of liver cirrhosis. Additionally, although TNF-α inhibitor worked well in our results of TNF-α and IL-6, liver damages did not be improved by PTX (Fig. 12). Previous researchers demonstrated that TNF-α itself will cause acute liver failure [31,32], but the inhibition of TNF-α did not improve liver damage in our model. We speculated that TNF-α was secondarily elevated in liver damage by DMN in our model, and this result and our data in vitro seem to be interesting that beneficial effects against hepatocytes damages of WHP mainly depend on hepatocyte protective effects, not on anti-inflammatory reaction after hepatocytes injury in the acute phase.

WHP also attenuated production of TGF-β, a key profibrogenic cytokine that promote to activate HSCs in fibrogenesis process. We evaluate the degree of myofibroblasts, including activated HSCs, by α-SMA staining. The results suggested that WHP prevent activation of hepatic stellate cells, which resulted in decreased hydroxyproline content in liver tissue.

We then examined hepatocyte damage using cultured hepatocytes; as it is known that hepatocyte damage is the first step leading to fibrosis before HSC activation. It is believed that continuous hepatocellular damage with remodeling, macrophage phagocytosis of necrotic hepatocytes, and extracellular matrix production by activated HSC lead to liver fibrosis [7,33, 34]. Two studies using cultured hepatocytes were conducted. The first investigated the hepatocyte-protective effect in vivo using hepatocytes isolated from rats fed a WHP diet. There were fewer necrotic hepatocytes after DMN challenge in rats fed the WHP-enriched diet compared with rats fed the control diet. This suggests that continuous WHP administration modified hepatocellular function to protect against DMN injury.

Finally, we investigated the hepatocyte-protective effect in vitro using hepatocytes with WHP-added medium directly. Hepatocellular injury after DMN challenge was dramatically suppressed in hepatocytes cultured in WHP-added medium compared with cells cultured in medium alone or in a control peptide-added medium. This suggests that addition of WHP directly exerts a hepatocyte-protective effect on primary hepatocytes.

DMN is metabolized by CYP2 E1, an ethanol-inducible CYP, in hepatocytes, and toxic metabolites from DMN lead to hepatotoxicity and mutagenesis [34–36]. We examined whether WHP regulated CYP2 E1 in our model (Fig. 7). The expression of CYP2 E1 mRNA was statistically significantly lower in the WHP group in the acute phase (Fig. 7A). The results suggest that WHP might down-regulate CYP2 E1 and restrain production of toxic metabolites.

We also examined MDA as a quantity of ROS using the thiobarbituric acid method and GSH levels in liver tissue. In the WHP group, MDA levels were observed to be significantly lower and GSH levels were significantly higher in both the acute and chronic phases. Researchers have suggested that the formation of ROS causes chronic liver injuries and hepatic fibrosis. Oxidative stress could be one of the major causes of liver damage, and may be involved in hepatic fibrogenesis by the stimulation of collagen gene expression [9]. Data from the present study suggests that

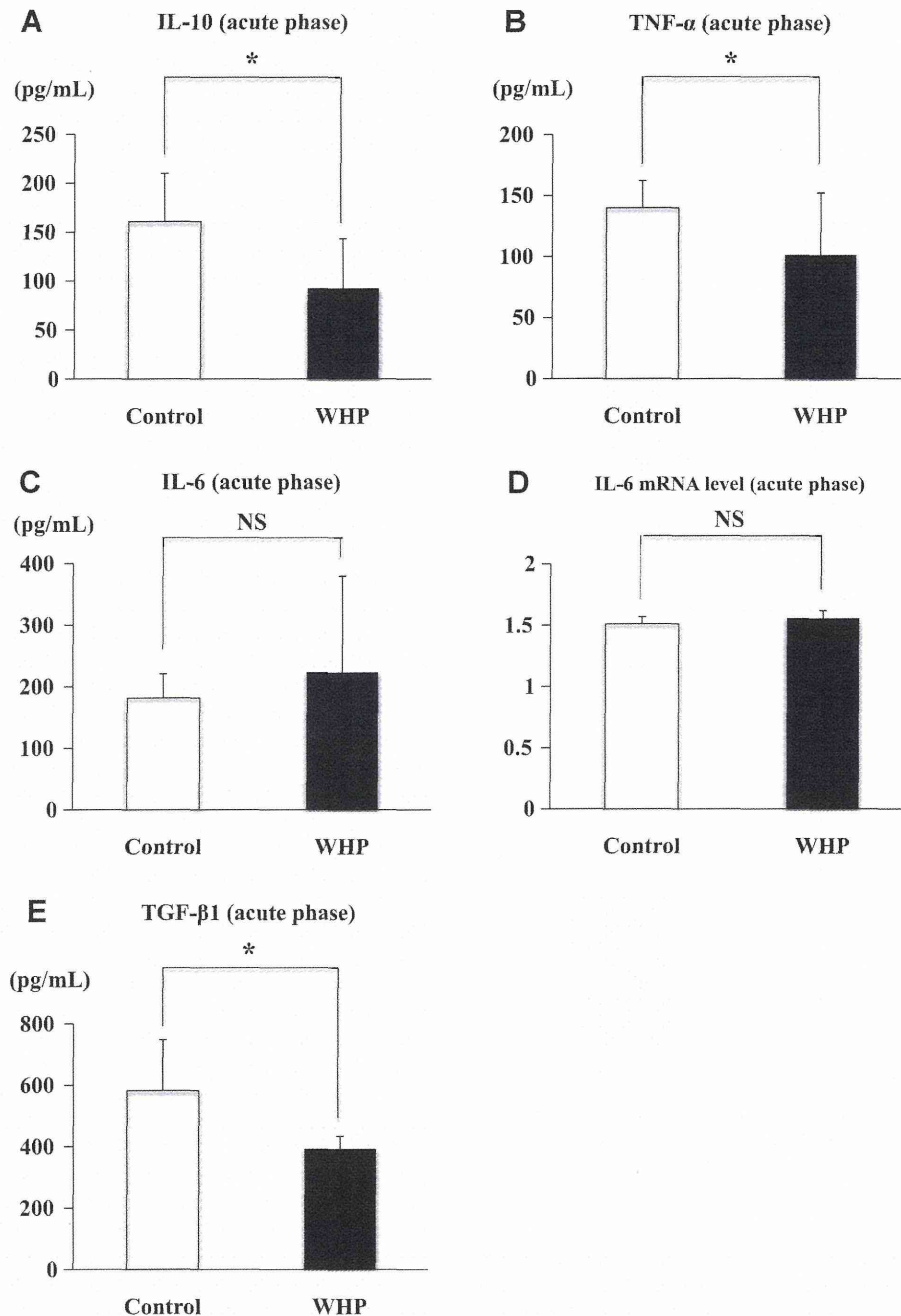


**Fig. 11.** Behaviors of inflammation-related cytokines in the acute phase. Serum IL-10 (A) and TNF-α (B) and TGF-β1 (E) levels were significantly lower in the WHP group than in the control group. Serum IL-6 levels and IL-6 mRNA levels in liver tissue showed no significant difference between groups (C, D). $^{*}P < 0.05$; NS, not significant ($P \geq 0.05$). IL, interleukin; PTX, pentoxyfilline; TGF, transforming growth factor; TNF, tumor necrosis factor, WHP, whey-hydrolyzed peptide.

the antioxidant effect of WHP protected hepatocytes and prevented liver fibrosis.

With regard to the mechanism of DMN toxicity on hepatocytes via the denitrosation metabolic pathway of DMN by CYP2 E1, nitric oxide (a type of ROS) is reported to be generated and injure hepatocytes [34,35]. We speculated that WHP first reduced ROS in the liver, and then showed hepatocyte-protective effects.

Previous researchers have reported that WHP has antioxidant activity, containing cysteine-rich domains that contribute in the synthesis of GSH, a potent intracellular antioxidant [11]. We found that GSH levels were higher with WHP intake, and this finding supports these studies. We speculate that one of the potential mechanisms for the hepatocyte-protective effects of WHP might be antioxidative actions through GSH synthesis. On the other hand, methionine promotes cysteine and GSH synthesis. This essential amino acid also shows antioxidant effects in the nearly same manner. Methionine-enrich diet may be another candidate for antifibrotic agents [37].

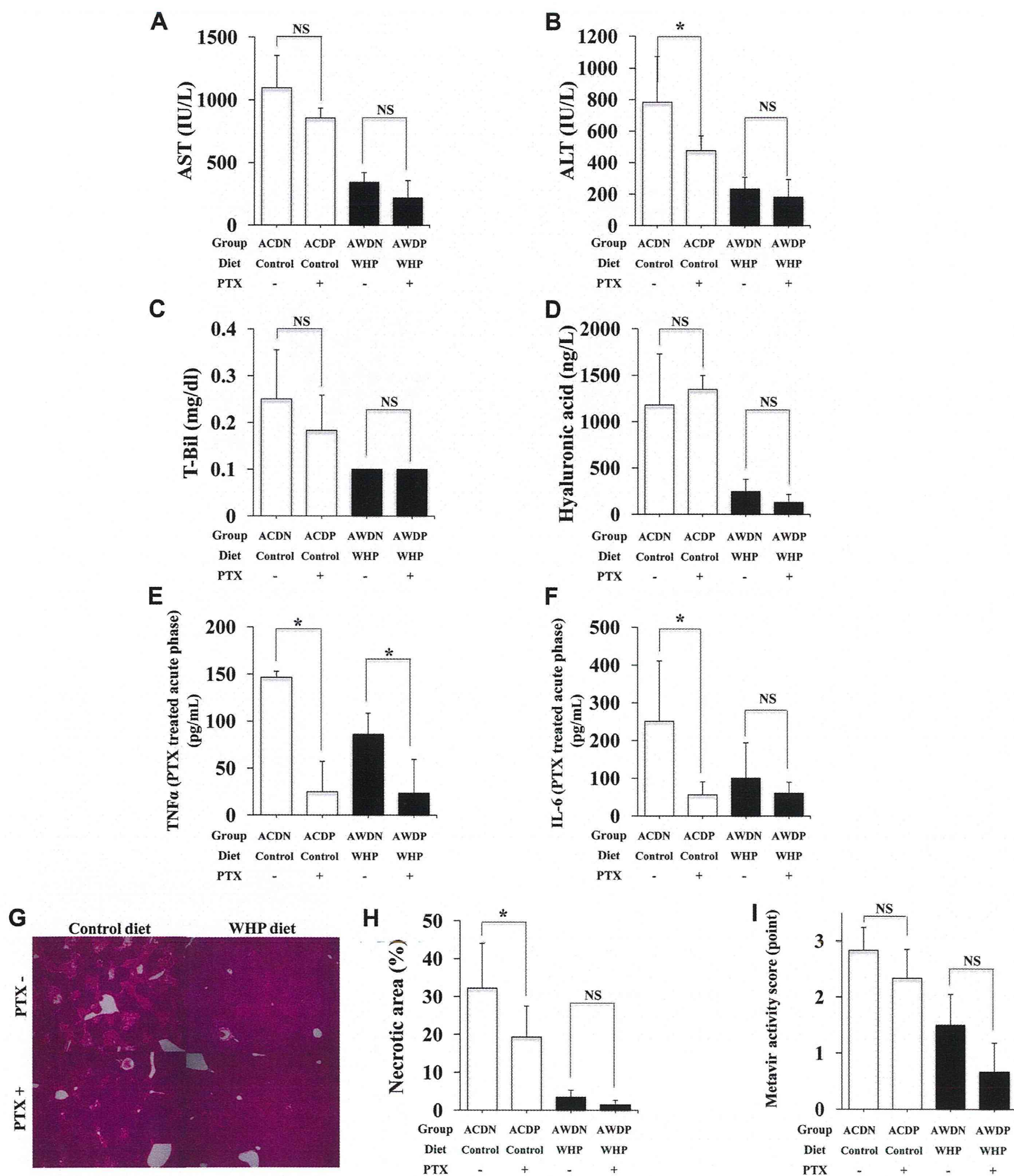


**Fig. 12.** Effects of TNF-α inhibitor in acute phase. TNF-α inhibitor (PTX) combined with WHP did not improve liver damages, though reduced significantly inflammatory cytokines (TNF-α and IL-6) in acute phase. *$P < 0.05$; NS, not significant ($P \geq 0.05$). ACDN, control diet without PTX; ACDP, control diet with PTX; AWDN, WHP diet without PTX; AWDP, WHP diet with PTX; IL, interleukin; PTX, pentoxyfilline; TNF, tumor necrosis factor; WHP, whey-hydrolyzed peptide.