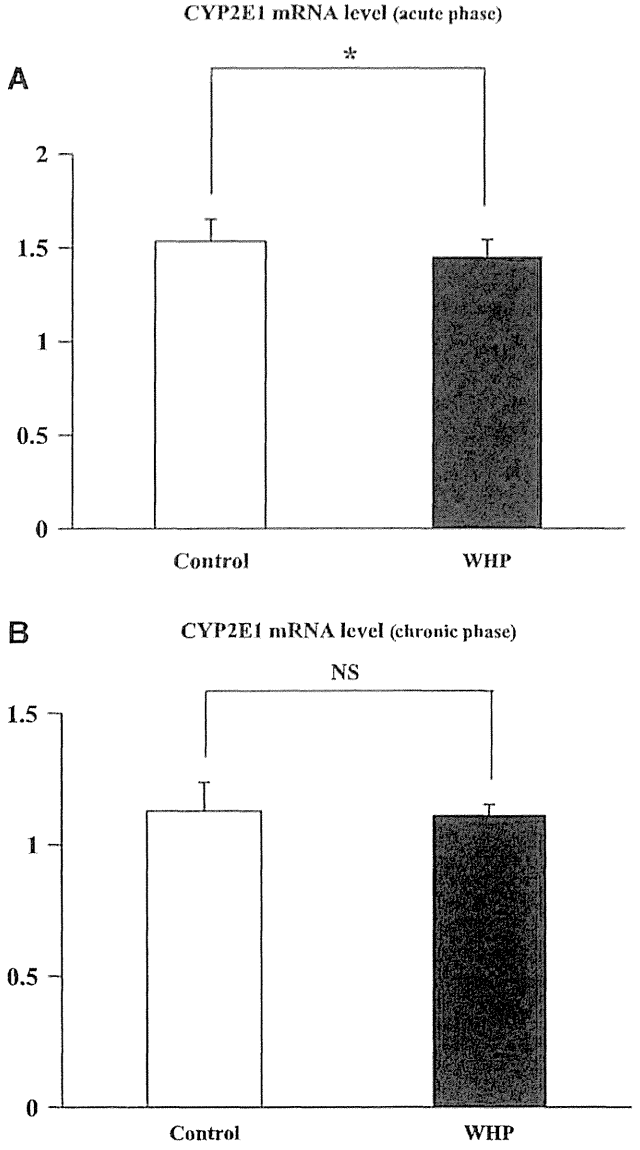

Fig. 8. Down-regulation of CYP2 E1 and restrained production of toxic metabolites by WHP treatment. In the chronic phase, CYP2 E1 mRNA levels showed no significant difference between the two groups (B). In the acute phase, CYP2 E1 mRNA levels were statistically significantly lower in the WHP group than 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 ±

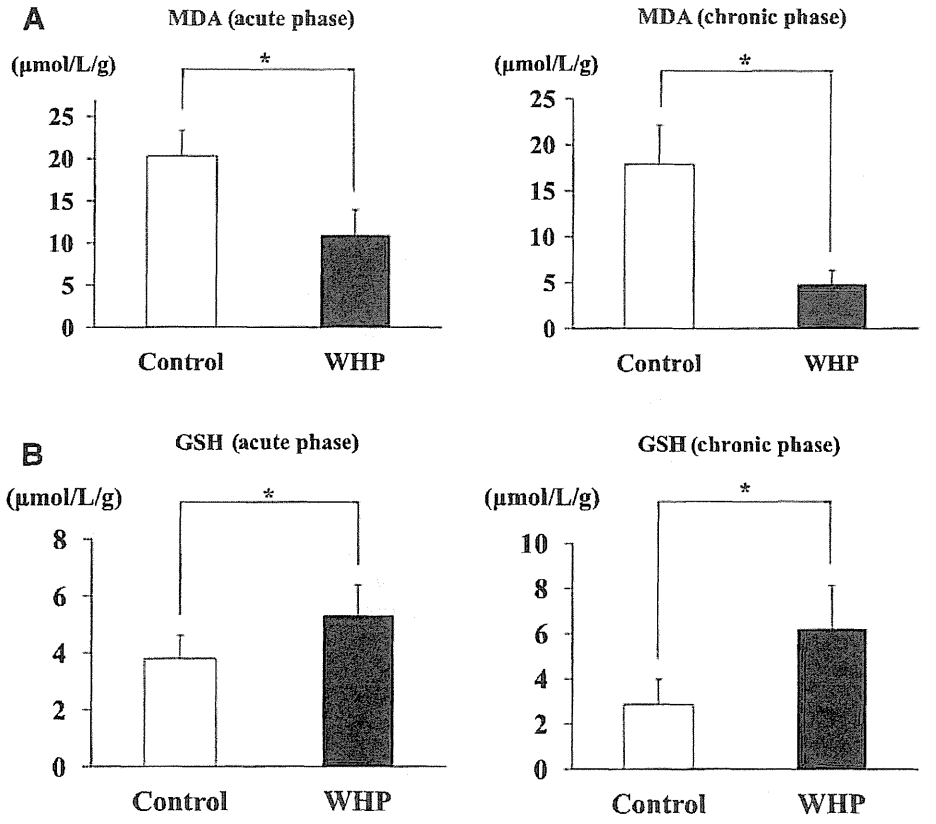

Fig. 9. Antioxidant effect of WHP to reduce ROS and keep GSH higher in liver. MDA levels, a quantitative variable of ROS, were significantly lower in the WHP group than in the control group in acute and chronic phase (A). GSH levels were significantly higher in the WHP group than those in control group (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

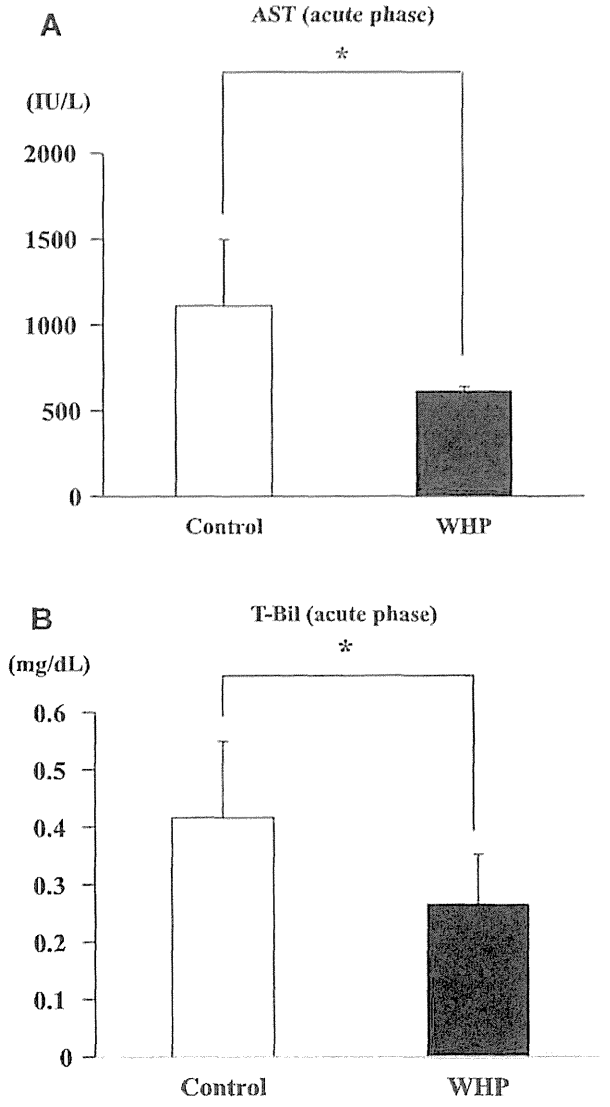

Fig. 10. Conventional liver function tests in the acute phase. AST and T-Bil levels were significantly attenuated in the WHP group. *$P < 0.05$. AST, aspartate aminotransferase; T-Bil, total bilirubin; WHP, whey-hydrolyzed peptide.

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

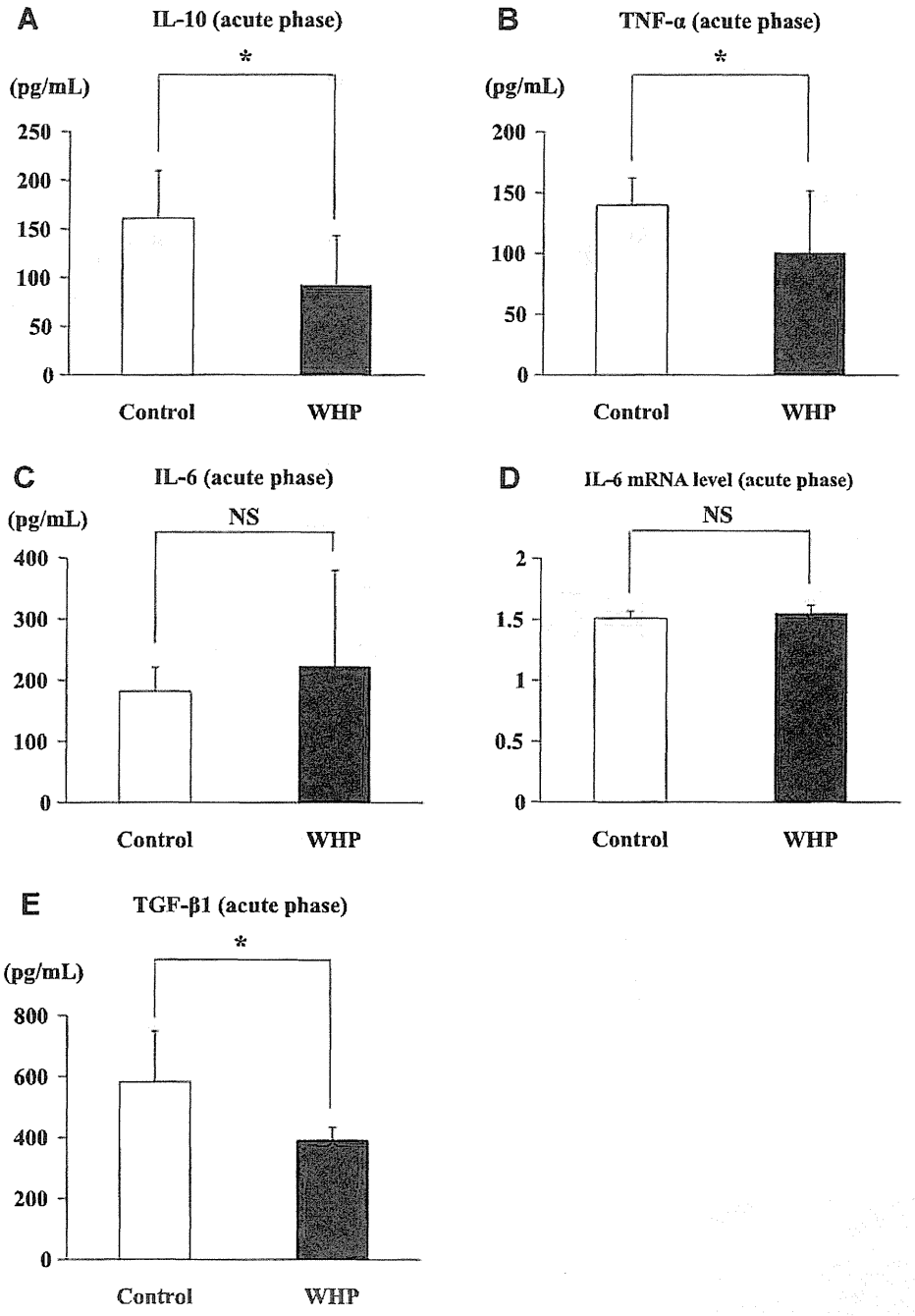

Fig. 11. Behaviors of inflammation-related cytokines in the acute phase. Serum IL-10 (A) and TNF-α (B) and TGF-β1 (E) levels were significantly lower in the WHP group than in the control group. Serum IL-6 levels and IL-6 mRNA levels in liver tissue showed no significant difference between groups (C, 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].

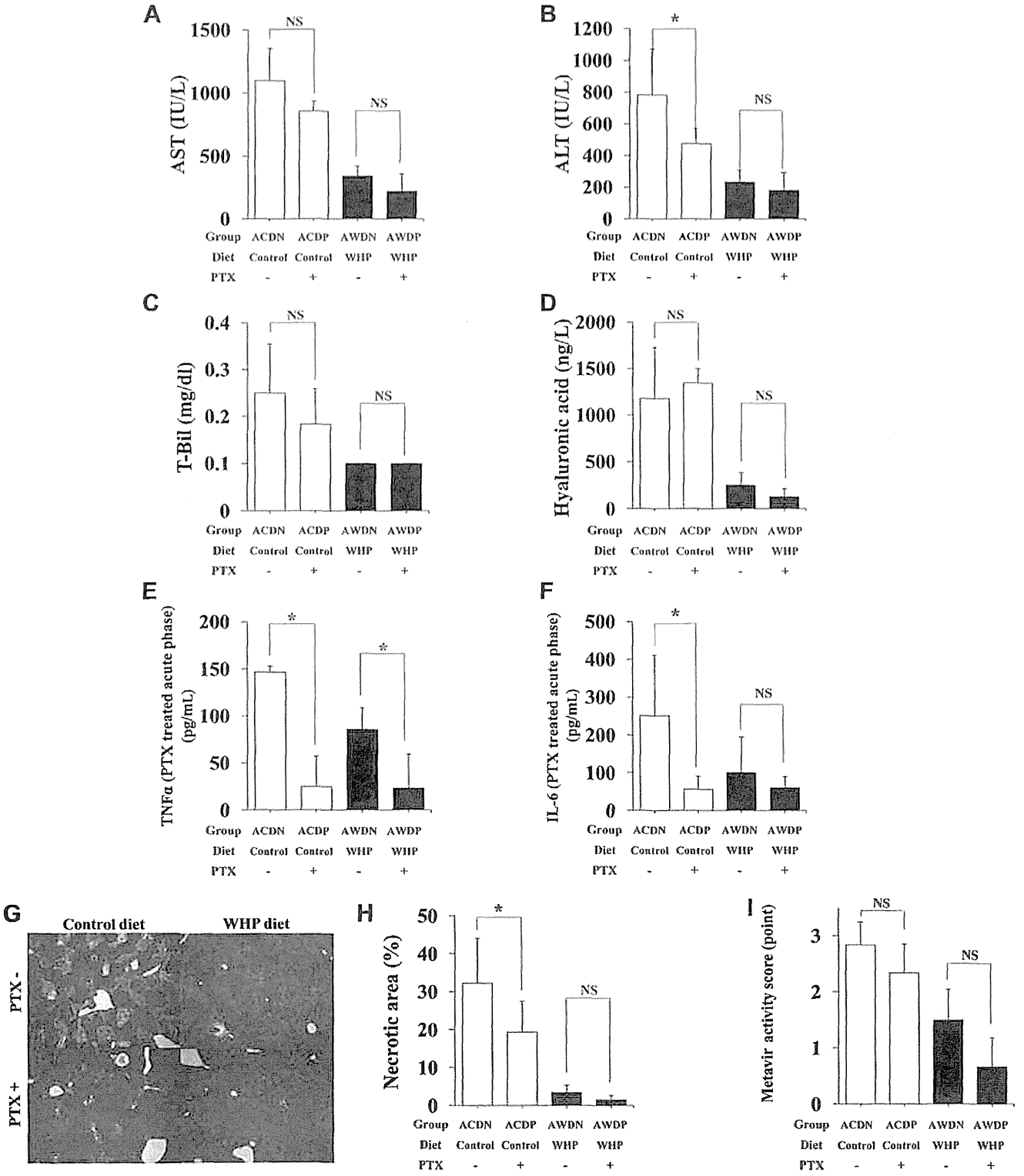

Fig. 12. Effects of TNF-α inhibitor in acute phase. TNF-α inhibitor (PTX) combined with WHP did not improve liver damages, though reduced significantly inflammatory cytokines (TNF-α and IL-6) in acute phase. *$P < 0.05$; NS, not significant ($P \geq 0.05$). ACDN, control diet without PTX; ACDP, control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

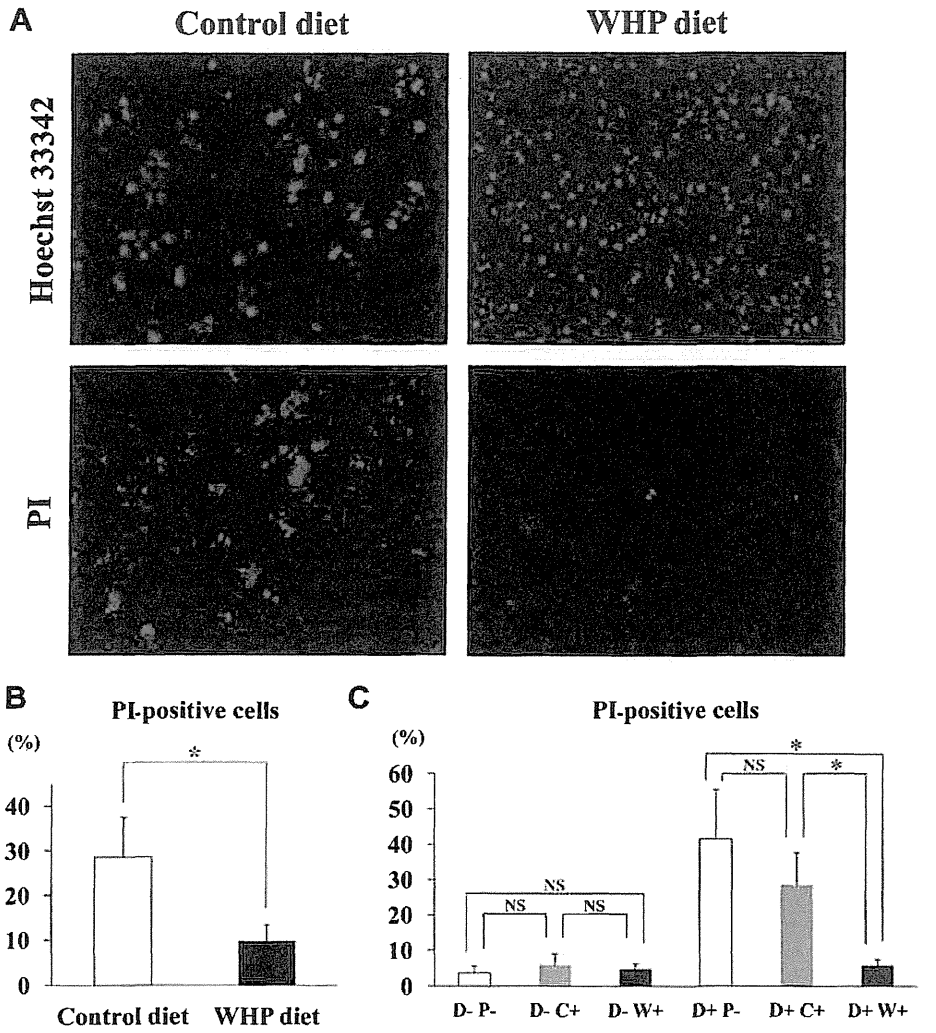

Fig. 13. Hepatocyte-protective effect of WHP. (A, B) Hepatocyte-protective effect in vivo for isolated hepatocytes from rats fed a WHP diet. The propidium iodide-positive ratios of cultured hepatocytes were significantly lower in the WHP diet group compared with the control diet group. (C) Hepatocyte-protective effect in vitro for hepatocytes with WHP-added medium. The propidium iodide-positive ratios were markedly lower in the D+W+ group compared with the D+P– group. *$P < 0.05$; NS, not significant ($P \geq 0.05$). PI, propidium iodide; WHP, whey-hydrolyzed peptide.

Conclusion

Our results clearly demonstrated that a WHP-enriched IMD effectively prevented DMN-induced liver fibrosis/cirrhosis in rats, possibly via a direct hepatocyte-protective effect and antioxidant effect through GSH synthesis. WHP may have therapeutic potential and be useful as an antifibrotic agent in the liver.

Acknowledgments

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