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Protective Effect of Cynanchum Wilfordii Root Crude Extract on Acetaminophen-Induced Liver Toxicity

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Abstract

The objective of this study was to investigate the protective effect of Cynanchum wilfordii root crude extract (CWR) on acetaminophen (AAP) overdose-induced liver damage. We tested the hepatoprotective effect of CWR in a normal liver cell line (Chang cell) using cell viability assay and western blot analysis. The protective effect of CWR was determined using hematoxylin and eosin staining and immunohistochemistry in vivo. Our results showed that the CWR significantly prevented AAP–induced cell death through the extra cellular signal-regulated kinase 1/2 (ERK1/2) activation. AAP-induced liver damage in mice was significantly reduced when CWR was administered at a dose of 100 mg/kg/day (for 7 days) in saline solution. CWR regulated the expressions of inflammation-related proteins such as tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS). These data suggest that CWR presents hepatoprotective effect via the activation of ERK1/2 and the down-regulation of TNF-α and iNOS, which are closely associated with anti-inflammatory responses. Moreover, these findings suggest that CWR can act as a potential therapeutic agent in the treatment of liver damage.

[Keywords] ***Cynanchum Wilfordii, Crude Extract, Liver, Acetaminophen, Anti-Inflammation***

1. Introduction

The liver is an essential organ that plays a vital metabolic role in detoxification and maintenance of blood glucose levels. Although this organ exhibits excellent regenerative capacity, it is highly vulnerable to chemical-induced damage and the accompanying oxidative stress. Liver failure, which is often associated with necrosis, is due to continuous and abnormal oxidative stress and drug-induced liver damage[1]. In western countries, approximately 50% of the total incidence of acute liver failure (ALF) is caused by drug abuse.

Acetaminophen (AAP), also known as paracetamol, has been widely prescribed for the treatment of high fever and pain. Yet, AAP can be harmful and lead to liver and kidney damage in children. Despite the fact that AAP is one of the leading causes of liver-related morbidity and mortality, most patients are unaware of the risks associated with AAP overdose[2][3]. Acetaminophen metabolism involves the formation of toxic intermediate by-products such as N-acetyl-p-benzoquinone imine (NAPQI), a highly reactive metabolite. In an acute overdose, this toxic metabolite induces inflammatory responses and hepatic necrosis[4].

Nitric oxide (NO) exhibits both harmful and beneficial functions in cellular metabolism. NO generation plays a crucial role of maintaining immune-homeostasis in cellular metabolism against bacterial infections. How-

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ever, they can also induce liver-cell dysfunctions because excessive NO generation induces cell death, cellular damage, and progression of diseases through the activation of immune responses[5].

Thus far, approximately 300 species of Cynanchum wilfordii belonging to the Cynanchum genus have been established. C. wilfordii species have been used in Korean traditional medicine to improve stamina[6]. Moreover, several studies have proven their effectiveness in preventing endothelial dysfunction, as well as their anti-fungal and antioxidant properties. However, the effect of C. wilfordii in maintaining the protection and regeneration in liver dysfunction has not yet been established. In this study, we investigated the effects of C. wilfordii in the regulation of cellular dysfunction as we expect that its combined treatment can be efficient in counteracting liver failure.

2. Methods

2.1. Chemicals

Cell culture reagents were purchased from Gibco BRL (Gaithersburg, MD). The EZ-cytox Cell Viability Assay and Vectastain ABC Kits were obtained from Daeil Lab Service (Seoul, Korea) and Vector Laboratories (Burlingame, CA, USA), respectively. The following chemicals and antibodies used in this study - COX-2, iNOS, TNF-α, APE1/Ref-1, and GAPDH - were purchased from Santa Cruz (Santa Cruz, MA). AAP and all other reagents were purchased from Sigma (St. Luis, MO).

2.2. Design of experiments

We performed the EZ-cytox Cell Viability Assay to evaluate cell viability, western blot to test the expression levels of proteins, and histopathological assay to determine the hepatoprotective effect of CWR.

2.3. Procedure

2.3.1. Plant materials and water extraction

C. wilfordii was obtained from Dongguk University Oriental Hospital, Korea. One-hundred grams of the plant root were blended, and the crude powder was precipitated with 1000 mL of sterile deionized water at 100°C for 3 h. The aqueous extracts were concentrated and evaporated at 60°C under vacuum conditions. The extract was dissolved in 50 mL of sterile deionized water. The aqueous extract was lyophilized by freeze-drying at -60°C. Finally, we obtained 27.4% powder (27.4 g) from the plant root.

2.3.2. Cell culture and cell viability assay

Chang cells were cultured in DMEM containing 10% FBS and 1% penicillin–streptomycin and maintained in a humidified atmosphere containing 5% CO2 at 37°C. A total of 5 × 104 cells were seeded in a 96-well microplate, and the cells were incubated with different concentrations of the C. wilfordii extract (CWR; dissolved and diluted in DMEM) for 24 h. Thereafter, cell viability was measured by carrying out ELSIA using the EZ-cytox Cell Viability Assay Kit, and the data were acquired using an ELISA reader according to the manufacturer's instructions.

2.3.3. Western blot

The cell lysates were obtained from the Chang cells after a defined culture period. Twenty micrograms of proteins were separated using SDS-PAGE on gels containing 12% acrylamide. Then, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech) at 4°C. This membrane was then soaked overnight in 5% skim milk at 4°C. Subsequently, the membrane was washed using Tris-buffered saline containing 0.1% Tween 20. The membrane was incubated with t-ERK1/2, p-ERK1/2 and GAPDH antibodies (1:1000 dilution). After incubation with the corresponding secondary antibodies, the membrane was analyzed using chemiluminescent reaction (ECL plus kit, Amersham Pharmacia Biotech), and proteins were visualized and analyzed using Image J Software.

2.3.4. Animal care

Six-week-old male ICR mice were obtained from Orient Bio. Inc., Korea. Prior to the experiments, all animals were allowed to acclimatize to the new environment for one week

(at room temperature, 24 ± 2 °C; humidity, 50 ± 15%; and 12-h light/dark cycle). All experiments and animal care were conducted in conformity with the institutional guidelines of the Dongguk University. To test the hepatocytoprotective effect of CWR, the animals were randomly divided into three different groups (n = 8 mice/group): control group (normal mice), CWR- and acetaminophen-treated group, and acetaminophen treated group. C. wilfordii root crude extract (CWR; 100 mg/kg/day in normal saline) was administered orally every day for 7 days, following a regular schedule. Liver injuries were induced by acetaminophen (diluted in normal saline). Acetaminophen was administered by a single intraperitoneal injection at a dose of 400 mg/kg, 1 h after the last CWR treatment.

2.3.5. Histological assay

The mice were anesthetized with sodium pentobarbital solution and killed. Their livers were isolated and fixed in 10% formalin for 24 h. The livers were embedded in paraffin, and 6-m-thick tissue sections were stained with hematoxylin-eosin (H&E) stain. Some sections were used for Wright's stain to observe blood cells in the liver. Some of the prepared sections were used for immunostaining assays. Samples were treated with 3% H2O2 for 5 min to inactivate endogenous peroxidase, then blocked with 10% normal serum for 1 h at room temperature, and incubated overnight at 4°C with primary antibodies against tumor necrosis factor-α (TNF-α) and inducible nitric oxide synthase (iNOS). The following day, the sections were washed and incubated with the corresponding secondary antibodies for 1 h at room temperature. The Vectastain ABC Kit was used to implement the avidin–biotin complex interaction, in accordance with the manufacturer's instructions. Signal development was carried out in a substrate solution of 0.05% DAB, and the slides were counterstained with hematoxylin. The sample sections were examined using a light microscope (Olympus BX50, Japan) at 200 X magnification.

2.3.6. Statistical analysis

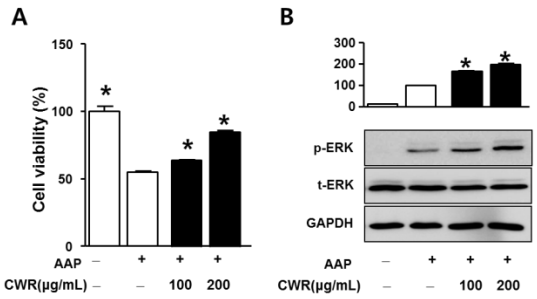
Data are expressed as the mean ± standard error of the mean (S.E.M.) in the indicated number of experiments. Statistical analysis of the data was performed using student's t test for comparisons between pairs of groups using GraphPad prism (GraphPad Software, San Diego, CA, USA). P values < 0.05 were considered significant.

3. Results

3.1. Effect of CWR on cell viability and ERK 1/2 phosphorylation in acetaminophen-stimulated Chang cells

We assessed whether CWR can regulate ERK1/2 phosphorylation in AAP-induced Chang cells. In this study, CWR showed no cytotoxicity at doses up to 200 μg/mL. AAP-stimulated Chang cells were treated with varying concentrations of CWR (100 and 200 μg/mL) for 24 h. As shown in <Figure 1A>, AAP significantly reduced cell viability, whereas CWR inhibited AAP-induced cell death in Chang cells. Next, the cell lysates were analyzed using western blot. Cell viability was significantly induced by CWR. The expression level of ERK1/2 phosphorylation was evaluated by normalizing the intensity of the bands against the control gene, ERK expression. As shown in <Figure 1B>, AAP-stimulated Chang cell treated with CWR at concentrations of 100 and 200 μg/mL significantly increased ERK1/2 phosphorylation to 150% and 190%, respectively.

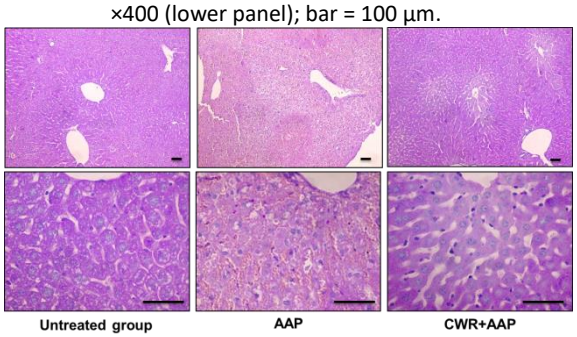
Figure 1. Effect of CWR on AAP-stimulated Chang cell (A) The cells were treated with the presence or absence AAP and CWR concentrations (100, and 200 μg/mL). Cell viability was determined via cell viability assay. (B) Cells lysates were examined via immunoblot analysis with specific antibodies such as anti-ERK1/2 phosphorylation (p-ERK), anti-total ERK1/2(t-ERK) and anti-GAPDH (GAPDH). The graphs represent the intensity of the bands relative to the AAP-treated group. Results are presented as means ± standard errors, and are representative of three independent experiments. *P < 0.05 versus untreated group.



3.2. Protective effect of CWR on acetaminophen-induced liver damage in mice

To examine the protective effect of CWR against acetaminophen-induced liver injury, we performed the histological analysis using H&E staining in a mouse model. In the group pretreated with CWR through oral administration (for 7 days) before overdosing with AAP, we observed decreased AAP-induced centrilobular degeneration and necrosis. As shown in <Figure 2>, tissue samples treated with acetaminophen (400 mg/kg) exhibited severe necrosis with vacuole formation in the hepatocytes. In contrast, samples pretreated with CWR (100 mg/kg) showed the morphology similar to normal naïve hepatocytes when compared to the untreated group.

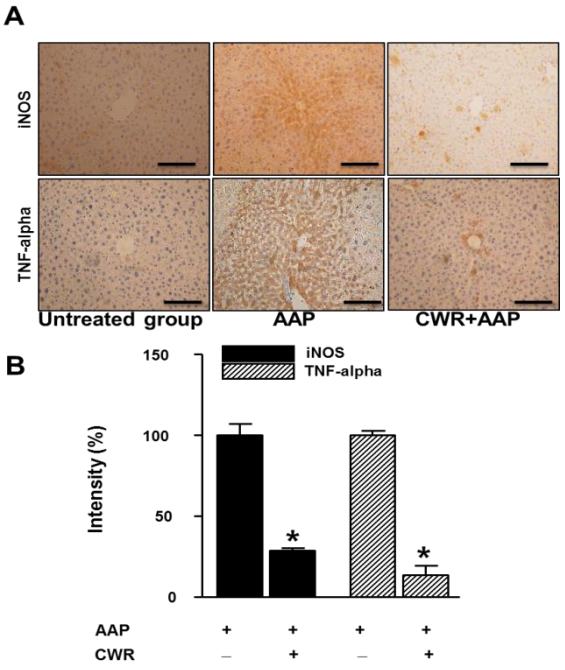
Figure 2. Effect of CWR in acetaminophen-induced liver injury in mice Liver sections were stained with H&E. The untreated group of animals was given water only (Untreated group), one group of animals was treated with acetaminophen (AAP), the other group was treated with acetaminophen after pretreatment with 100 mg/kg of CWR for 7 day (AAP+CWR). The photomicrographs show a magnification of ×200 (upper panel) and ×400 (lower panel); bar = 100 μm.



3.3. Effect of CWR on iNOS and TNF-α expression in AAP-induced injury of liver tissue

To examine possible correlations between hepatocyte protection and regulation of redox, we studied the expression levels of inflammation-related signaling molecules, iNOS and TNF-α, in AAP-induced liver failure in mice. We observed the expression levels of iNOS and TNF-α to be 100% in the AAP-treated group. CWR pretreatment decreased their expression by 70%, and 80% respectively.

Figure 3. Expression of iNOS and TNF-α in acetaminophen-induced liver failure and CWR-treated liver (A). The graph shows the expression levels of iNOS and TNF-α obtained from A. Expression changes are relative to the acetaminophen treated group (B). The results are representative of more than three independent experiments. Results are presented as the means ± standard errors, * $P < 0.05$ versus acetaminophen-treated group.



4. Discussion and Conclusion

Reactive oxygen species (ROS) are bifunctional catalyst molecules exhibiting both beneficial and harmful effects on the cells. ROS can support vital cellular activities such as induction of transcription factors and accommodation of the receptor signals. However, an excessively increased levels of ROS in the

cells lead to oxidative stress that can induce extensive cell damage such as DNA destruction, lipid peroxidation, and protein degeneration[7]. Overdose of AAP is associated with the ROS generation signals[1]. Dahlin et al. supported the idea that following acetaminophen overdose, AAP metabolism leads to the formation of a toxic intermediate such as N-acetyl-p-benzoquinone imine (NAPQI), which causes acute liver failure[3]. Furthermore, over dose of AAP induces cell death and ERK1/2 phosphorylation in Chang cells[8]. Especially, a defense mechanism is associated with ERK1/2 activation. We also confirmed that CWR regulates ERK 1/2 phosphorylation. Therefore, we suggest that CWR may have an antioxidant effect.

AAP overdose-induced liver damage is highly relevant to the inflammatory signaling pathway that includes components such as TNF-α signal and pro-inflammatory cytokines[2][5]. In particular, up-regulation of TNF-α and expression of iNOS have been observed in histological sections of liver lesions. The signal cascades and transductions are triggered by phosphorylation of proteins. Especially, the extracellular signal-regulated kinases 1/2 (ERK1/2), which are activated by cell survival signal, participate in liver damage and regeneration [6]. We also confirmed that CWR regulates TNF-α and iNOS. The results suggest that CWR is one of the candidates for combined prescription against AAP-induced liver damage and can significantly regenerate liver injuries.

In conclusion, we clearly demonstrate the hepatocyte-protective effect of CWR in vitro and vivo. Our results suggest that CWR down-regulates ERK1/2 phosphorylation that is attributable not only to the suppression of iNOS but also to the regulation of TNF-α expression. Therefore, CWR is a promising therapeutic agent against acute and chronic liver failure induced by AAP over-dosage.

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