Hepatoprotective Activity of Yellow Chinese Chive against Acetaminophen-Induced Acute Liver Injury via Nrf2 Signaling Pathway

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Summary Glutathione, the most abundant intracellular antioxidant, protects cells against reactive oxygen species induced oxidative stress and regulates intracellular redox status. We previously demonstrated that yellow Chinese chive (ki-nira) increased the intracellular glutathione levels. Acetaminophen (APAP) is a commonly used analgesic. However, an overdose of APAP causes severe hepatotoxicity via depletion of the hepatic glutathione. In this study, we investigated the hepatoprotective effects of yellow Chinese chive extract (YCE) against APAP-induced hepatotoxicity in mice. YCE (25 or 100 mg/kg) was administered once daily for 7 d, and then APAP (700 mg/kg) was injected at 6 h before the mice were sacrificed. APAP treatment markedly increased the serum biological markers of liver injury such as alanine aminotransferase, aspartate aminotransferase, lactate dehydrogenase, and alkaline phosphatase. Pretreatment with YCE significantly prevented the increases in the serum levels of these enzymes. Histopathological evaluation of the livers also revealed that YCE prevented APAP-induced centrilobular necrosis. Pretreatment with YCE dose-dependently elevated glutathione levels, but the difference was not significant. Nuclear factor erythroid 2-related factor 2 (Nrf2) plays a critical role in APAP-induced hepatotoxicity by regulating the antioxidant defense system. Therefore, we investigated the expression of Nrf2 and its target antioxidant enzymes. YCE led to an increased expression of Nrf2 and its target antioxidant enzymes, NAD(P)H quinone oxidoreductase 1 (NQO1), glutathione peroxidase (GPx), cystine uptake transporter (xCT), especially hemeoxygenase-1 (HO-1) in mice livers. These results suggest that YCE could induce HO-1 expression via activation of the Nrf2 antioxidant pathway, and protect against APAP-induced hepatotoxicity in mice.

Key Words yellow Chinese chive, acetaminophen, hepatotoxicity, nuclear factor erythroid 2-related factor 2 (Nrf2), hemeoxygenase-1 (HO-1)

Yellow Chinese chive, named ki-nira in Japan, is a vegetable often used in Japan and China. Yellow Chinese chives and Chinese chives (green vegetable), both used as leafy vegetable, are the same varieties (Allium tuberosum Rottler) but their cultivation methods differ. Yellow Chinese chives are cultivated in the shade under covering materials by regrowing after cutting at the lower part, to keep them out of direct sunlight. Yellow Chinese chives produced in Okayama Prefecture account for 70% of the Japanese yield. Previous research demonstrated that Chinese chive exhibited antioxidant (1), antitumor (2), antidiabetic (3), hair growth promoting (4), and renoprotective (5) effects. Although Chinese chive has been reported to possess several bioactivities, not much has been reported on the bioactivities of yellow Chinese chive. In a previous study, we demonstrated that yellow Chinese chive increased intracellular glutathione levels (6).

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Glutathione (γ-glutamyl-L-cysteinyl-glycine) is an important thiol compound with antioxidant activity; it plays important roles in counteracting oxidative stress injury and maintaining cellular redox balance (7). Altered glutathione metabolism and the resulting increase in oxidative stress have been implicated in the pathogenesis of several diseases such as protein energy malnutrition, seizures, Alzheimer’s disease, Parkinson’s disease, sickle cell anemia, chronic diseases associated with ageing, and the infected state (8, 9). Therefore, regulating glutathione biosynthesis may be a useful approach to prevent such diseases.

Acetaminophen (APAP) is one of the most commonly used analgesic and antipyretic drugs worldwide. Within the range of therapeutic dose, a majority of APAP is metabolized by cytochrome P450 to form the highly reactive species, N-acetyl-p-benzoquinone imine (NAPQI), which can be rapidly detoxified by conjugation with glutathione under normal conditions. However, an overdose of APAP causes an increase in NAPQI levels; NAPQI covalently modifies liver protein and depletes the
intracellular glutathione. The binding of NAPQI to cellular macromolecules induces oxidative stress, ultimately leading to apoptosis and hepatic necrosis \((10, 11)\). Thus, inhibition of oxidative stress is considered an important strategy for treating APAP-induced hepatoxicity.

Previous studies have demonstrated that nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor that regulates the expression of cytoprotective genes such as NAD(P)H quinone oxidoreductase 1 (NQO1), glutathione peroxidase (GPx), cystine uptake transporter (xCT), hemeoxygenase-1 (HO-1), and \(\gamma\)-glutamylcysteine synthetase (\(\gamma\)-GCS) \((12, 13)\). We previously demonstrated that rice-derived peptides restored the expression of \(\gamma\)-GCS in mice with APAP-induced liver injury in addition to restoring glutathione levels \((14)\). A previous study reported that induction of HO-1 expression contributes to protection against liver damage induced by APAP \((15)\). In addition, Nrf2-deficient mice have been reported to exhibit increased sensitivity to APAP \((16)\). Thus, Nrf2 is considered a potential therapeutic target to prevent liver injury \((17)\).

In this study, we evaluated the protective effect of yellow Chinese chive against APAP-induced liver injury in mice. We also investigated whether yellow Chinese chive regulates the Nrf2 signaling pathway.

**MATERIALS AND METHODS**

**Materials.** Yellow Chinese chive was obtained from JA Okayama, Okayama, Japan and stored at \(-30^\circ\text{C}\) until use. Bathophenanthroline disulfonic acid, disodium salt (BAPS) was purchased from Dojindo Laboratories, Kumamoto, Japan. RIP A buffer was obtained from Santa Cruz Biotechnology, Santa Cruz, CA, USA. Polyvinylidene fluoride (PVDF) membrane was purchased from Bio-Rad, Hercules, CA, USA. PVDF Blocking Reagent was purchased from Toyobo, Osaka, Japan. Antibodies against GPx, xCT, \(\gamma\)-GCS, and Nrf2 were purchased from Abcam, Cambridge, United Kingdom. Anti-NQO1 was purchased from Cell Signaling Technology, Beverly, MA, USA. Anti-HO-1 was purchased from Enzo Life Sciences, Farmingdale, NY, USA. Anti-\(\beta\)-actin was purchased from Sigma-Aldrich, St. Louis, MO, USA. Horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibodies and ECL reaction solution were obtained from GE Healthcare, Buckinghamshire, United Kingdom.

**Preparation of yellow Chinese chive extract (YCE).** YCE was prepared as previously described \((18)\). Frozen yellow Chinese chive (200 g) were minced in a food processor and extracted with 50% aqueous ethanol (600 mL) at room temperature (23–25\(^{\circ}\text{C}\)) for 1 h. The liquid phase was evaporated in vacuo and lyophilized using a freeze-dryer to obtain a yellow residue (6.4 g).

**Treatment of animals.** Four-week-old male ICR mice were purchased from Japan SLC, INC., Shizuoka, Japan. The animals were housed at 24±1\(^{\circ}\text{C}\) with a 12 h shift in the light/dark cycle and had free access to a standard diet (MF; Oriental Yeast Co., Ltd., Osaka, Japan) and distilled water for 1 wk before the experiment. Mice were randomly assigned to five groups; group 1: control without treatment, group 2: YCE (100 mg/kg) treatment, group 3: APAP treatment, group 4: APAP+YCE (25 mg/kg) treatment, and group 5: APAP+YCE (100 mg/kg) treatment. The YCE treatment groups were orally administered YCE (25 or 100 mg/kg body weight) daily for 7 d. The control and APAP groups were administrated saline. All mice were fasted 18 h before intraperitoneal injection of APAP (700 mg/kg body weight) and

| Table 1. Effect of YCE on body weight and hepatic glutathione levels in mice with liver injury induced by APAP. |

| Groups          | Body weight (g) | Total glutathione (mmol/g liver) |
|-----------------|-----------------|----------------------------------|
| Control         | 33.7±0.8        | 3.4±0.1                          |
| YCE 100 mg/kg   | 34.3±1.0        | 3.6±0.1                          |
| APAP            | 33.2±0.4        | 0.16±0.05###                    |
| APAP+YCE 25 mg/kg | 34.0±0.6     | 0.20±0.03###                    |
| APAP+YCE 100 mg/kg | 33.3±0.6    | 0.36±0.08##                    |

Values are mean±SE \((n=5)\). ### \(p<0.01\) vs. control group.
sacrificed 6 h after APAP treatment. Blood samples were collected from the inferior vena cava under isoflurane anesthesia. For coagulation to occur, the blood was maintained at room temperature for 30 min, and then centrifuged at 750 × g for 10 min at 4°C. Livers were collected from each mouse for western blotting and histopathological analysis. All animal experimental protocols were approved by the Animal Experimentation Committee of Shujitsu University (Permit Number: 028-002); the study was conducted in accordance with the Animal Experimentation Guidelines of Shujitsu University.

Biochemical analysis. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using a Transaminase CII Test Wako kit (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). The results are expressed in Karmen units. Lactate dehydrogenase (LDH) and alkaline phosphatase (ALP) were measured using Cytotoxicity Detection KitPLUS (Roche Applied Science, Mannheim, Germany) and LabAssay ALP (FUJIFILM Wako Pure Chemical Corporation), respectively.

Histopathological analysis of liver. Liver tissues were fixed in 10% phosphate buffered neutral formalin, dehydrated with a graded series of alcohol, embedded in paraffin, and sectioned at 6 μm. Thin sections were stained with hematoxylin and eosin (H&E), and viewed under a light microscope (Keyence, Osaka, Japan).

Detection of glutathione. Total glutathione (reduced and oxidized form) levels were measured using a previously described method (19). The liver tissue (0.1 g) was homogenized in 10-fold 0.1 M HCl containing 1 mM BAPS. After deproteinization, the resulting supernatants were used to measure the total glutathione content. The concentration of total glutathione was expressed as μmol/g liver.

Western blotting analysis. Protein samples were prepared from the liver tissue, which was homogenized in RIPA buffer. After incubation for 30 min at 4°C, the homogenates were centrifuged at 18,800 × g for 10 min at 4°C. The protein concentration of tissue homogenates was determined by the BioRad protein assay. For immunoblot analysis, proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred electrophoretically to a PVDF membrane. The membrane was then blocked with...
Immunoblotting was performed using primary antibodies against γ-GCS, xCT, NQO1, GPx, HO-1, and Nrf2 overnight at 4°C. Then, appropriate secondary peroxidase-conjugated anti-rabbit or anti-mouse IgG were applied at room temperature for 1 h. Target proteins were visualized using an ECL reaction solution. β-Actin was used as internal control. The relative densities of target proteins were quantified using Image J software (National Institutes of Health, MD, USA).

**Statistical analysis.** All data are expressed as the mean ± standard error of the mean (SE). Data were statistically analyzed using ANOVA followed by the Dunnett’s test to determine significance between groups. p values lower than 0.05 were considered statistically significant.

**RESULTS**

**Effect of YCE against APAP-induced liver injury in mice**

To assess the damage in the mouse liver treated with APAP and YCE, several serum biochemical markers (AST, ALT, LDH, and ALP) associated with liver failure were measured. The final body weight was not significantly different among the five groups (Table 1). Administration of YCE alone did not affect serum levels of these markers. Basal serum levels of AST (Fig. 1A) and ALT (Fig. 1B) in the control group were 27.6 ± 2.1 and 7.7 ± 0.5 Karmen units, respectively, and treatment with APAP for 6 h markedly increased AST and ALT levels to 3,544.2 ± 259.7 and 1,229.6 ± 107.3 Karmen units, respectively, whereas the respective values for the control group were 4.1 ± 0.5 U/L and 80.8 ± 4.7 U/mL. In contrast, pretreatment with YCE significantly inhibited the elevation of serum AST, ALT, LDH, and ALP levels.

Glutathione plays an important role in scavenging NAPQI, a toxic metabolite of APAP (20). APAP treatment induced glutathione depletion (Table 1). Pretreatment with YCE dose-dependently elevated glutathione levels, but the difference was not significant.

Histological examination of the livers from the control (Fig. 2A) and YCE only-treated mice (Fig. 2B) showed...
normal lobular architecture and cell structure. APAP treatment induced hepatocellular necrosis, and vacuolations in the hepatocytes and sinusoidal congestion (Fig. 2C). YCE pretreatment showed a moderate degree of necrosis and vacuolations in the hepatocytes with minimal sinusoidal congestion (Fig. 2D, E). These results indicate that YCE protects against APAP-induced liver injury.

**Effect of YCE treatment on γ-GCS, xCT, NQO1, GPx, HO-1, and Nrf2 protein expression levels in the liver of APAP-treated mice**

Nrf2 and its downstream phase II detoxifying/antioxidant enzymes are considered to protect cells against free radical damage (21). We observed whether the protective effect of YCE against APAP-induced liver injury is associated with Nrf2 and phase II detoxifying/antioxidant enzymes. APAP treatment significantly decreased γ-GCS, NQO1, GPx, and Nrf2 protein expression compared to that in the control group (Fig. 3A, lane 3). HO-1 protein expression increased in the APAP-treated group compared to that in the control group. On the contrary, YCE dose-dependently increased the protein expression levels of xCT, NQO1, GPx, HO-1, and Nrf2 compared to that in the APAP-treated group (Fig. 3B). YCE pretreatment restored the protein expression of xCT, NQO1, and Nrf2 to the same level as that in the control group. No significant difference in γ-GCS protein expression was observed between the APAP-treated and YCE-treated groups. Pretreatment with YCE alone did not increase the expression levels of these proteins compared to those in the control group. These results suggest that the protective effect of YCE may be mediated through activation of the Nrf2 antioxidant pathway.

**DISCUSSION**

APAP-induced hepatotoxicity has served as the most popular, mechanistically well studied and clinically relevant liver injury model for testing potential hepatoprotective candidates (22). Recently, extensive efforts have been made to discover hepatoprotective agents from natural products in an effort to lower side effects (17). In the present study, we assessed the effect of oral administration of YCE on APAP-induced liver injury. APAP caused a significant increase in the serum levels of AST, ALT, LDH and ALP (Fig. 1); however, pretreatment with YCE significantly decreased these levels. Moreover, results from histological observations of the H&E-stained liver sections showed that YCE significantly decreased hepatic necrosis (Fig. 2). Thereby, we showed that YCE protects liver tissue against APAP-induced damage.

Excessive formation of NAPQI by overdose of APAP can lead to the depletion of hepatic glutathione (23). Glutathione plays an important role in the antioxidant defense system and may serve as a key determinant of APAP-induced hepatotoxicity. Accordingly, we measured hepatic glutathione levels. YCE dose-dependently elevated glutathione levels, but no significant differences in glutathione levels were observed between the APAP- and YCE-treated groups (Table 1). We further determined whether YCE could upregulate glutathione biosynthesis. γ-GCS catalyzes the first and rate-limiting step in glutathione biosynthesis (24, 25). In this study, we observed that YCE did not affect γ-GCS expression in mice liver (Fig. 3B). Expression of xCT on the cell membrane is essential for the uptake of cysteine required for intracellular glutathione synthesis, which plays an important role in maintaining the intracellular redox balance (26, 27). Our results showed that YCE treatment significantly increased the expression of xCT (Fig. 3B). These results suggest that YCE may accelerate hepatic glutathione recovery by inducing the cysteine uptake.

Nrf2 regulates the expression of phase II detoxifying/antioxidant enzymes such as γ-GCS, xCT, NQO1, GPx, and HO-1, which counteract oxidative stress by enhancing the removal of reactive oxygen species (ROS). NQO1 is an important enzyme for detoxification, because it reduces a broad range of chemically reactive quinones and quinoneimines. A previous study showed that NQO1 plays a critical role in the improvement of mitochondrial dysfunction caused by APAP (28). In addition, NQO1-mediated reduction is an effective pathway to detoxify quinoneimine metabolites such as NAPQI in addition to glutathione conjugation (29). Our results indicated that YCE induced the expression of NQO1 compared with APAP treatment (Fig. 3B). The production of ROS including hydrogen peroxide, hydroxyl radicals, and superoxide anions can be enhanced by NAPQI. Lipid peroxidation, DNA oxidation, and protein oxidation, as well as a decrease in an antioxidant enzyme of GPx have also been reported in APAP-induced liver injury (30, 31). GPx catalyzed the conversion of glutathione from the reduced form to the oxidized form, and the reduction of peroxides (ROOH: including H₂O₂) into alcohol (ROH) (32). Compared to the APAP-treated group, YCE significantly increased the expression of GPx (Fig. 3B). The effects of YCE on GPx activity are not yet known. The induction of GPx by YCE could reduce liver damage by scavenging the hydroxyl radicals generated by APAP.

Compared with the control group, APAP treatment decreased the expression of Nrf2 and its downstream target proteins, including γ-GCS, NQO1, and GPx. However, APAP treatment increased HO-1 protein expression. Our results are in agreement with those reported by Dkhil et al. (33) and Wu et al. (34). Wu et al. reported that APAP treatment increased the expression of the nuclear factor κB (NFκB). Activation of NFκB reportedly induces HO-1 expression (35). Thus, the induction of HO-1 by APAP may also be mediated via NFκB activation.

HO-1 expression contributes to protection against liver damage induced by APAP, suggesting HO-1 induction as an important cellular mechanism for hepatoprotection (15). The main function of HO-1 is to degrade heme and generate carbon monoxide (CO) and biliverdin which are both powerful antioxidants (36). According to previous reports, sulforaphane (37), sake lees hydrolysate (38), and salvianolic acid C (34) play protective roles against APAP-induced hepatotoxicity.
though antioxidant effects mediated by HO-1 induction. YCE induced HO-1 expression in mice livers (Fig. 3B). Compared with the APAP-treated group, there was a marked increase of 3.0-fold of the protein expression level of HO-1 in YCE (100 mg/kg)-treated group. Nrf2 is an important transcription factor, which has been reported to play a key protective role against APAP-induced liver injury (39). The decreased expression of Nrf2 in APAP-treated mice was restored by YCE treatment (Fig. 3B). Our data suggest that YCE activates Nrf2, which then enhances the expression especially of HO-1, and contributes to the protective effect of YCE against APAP-induced liver injury.

Normally, Nrf2 is localized in the cytoplasm where it interacts with the actin-binding protein, Kelch-like ECH associated protein 1 (Keap1) and is rapidly degraded by the ubiquitin-proteasome pathway. Upon electrophilic/oxidative stress, Nrf2 is dissociated from Keap1 and translocates to the nucleus (40). Previously, studies have reported that Chinese chives include sulfur-containing compounds, such as allyl methyl disulfide, diallyl disulfide, allyl methyl trisulfide, dimethyl trisulfide, and diallyl trisulfide (41, 42). Among them, diallyl trisulfides reportedly induce the transcriptional activation of Nrf2 and subsequently express HO-1 and NQO1 by directly modifying Keap1 (43). Thus, active ingredients of YCE are possibly sulfur-containing constituents. As the cultivation method of yellow Chinese chives differs from that of Chinese chives, the content of sulfur-containing compounds and other ingredients may also differ. Further studies are necessary to clarify the bioactive compounds and the mechanisms of Nrf2 activation.

In conclusion, we have demonstrated that YCE has a potent hepatoprotective effect against APAP-induced liver injury in mice. Our results show that the hepatoprotective effect of YCE was associated with antioxidative properties through activation of the Nrf2 signaling pathway. Thereby, we suggest that YCE may be a beneficial food or supplement to protect against liver injury.

Authorship
K.K., C.M., and S.T. designed the experiments. K.K., C.M., and S.T. performed the experiments. K.K. wrote the manuscript. K.K., C.M., T.H., E.S., and S.T. discussed the results, commented on the manuscript, and approved the manuscript submission.

Disclosure of state of COI
No conflicts of interest to be declared.

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