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Effect of Ash Tree Leaf Extract on Acetaminophen-Induced Hepatotoxicity in Mice

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Abstract This study was carried out to investigate the effects of ash tree leaf extract (ALE) on acetaminophen (APAP)-induced hepatotoxicity in mice. Hepatoprotective effects were detected by biochemical analysis of hepatic enzymes and histopathological examination of the liver. BALB/c mice were divided into three groups: 'normal' control mice, APAP-treated control mice, and mice pretreated with ALE and treated with APAP. A single dose of APAP markedly increased levels of plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Light micrographs of liver cells stained with hematoxylin and eosin showed that APAP induced severe centrilobular necrosis, degeneration, and infiltration by inflammatory cells. Moreover, APAP caused the numbers of TUNEL-positive hepatocytes to increase and caused glycogen content to decrease as observed by Periodic acid-Schiff stain. However, pretreatment with ALE for 7 days prior to the administration of APAP significantly decreased plasma levels of AST and ALT. Histological findings demonstrated that ALE pretreatment alleviated APAP-induced liver damage, and induced the regeneration of liver tissue and restoration of glycogen. These results indicate that ash tree leaf extract exerts a protective effect against APAP-hepatotoxicity induced injury.

Keywords: acetaminophen, ash tree leaves, hepatotoxicity, histopathology

Introduction

The liver is the key organ for metabolism and detoxification. Continuous exposure to a variety of toxic environmental agents can result in hepatic injury. Interest has grown worldwide in medicinal plants as therapeutic agents for the prevention of such injury. They are relatively safe and there is increasing evidence of their physiological properties and therapeutic effects in animals and humans (1, 2).

Ash trees (*Fraxinus rhynchophylla*) are often been planted as ornamentals in urban areas throughout Asia, Europe, and North America. The stem bark of the ash tree, a commonly used Chinese herbal drug, has been proven to be effective in the treatment of diarrhea and dysentery caused by intense heat. It has been reported to be especially effective for dysentery accompanied by bloody stools and for lung disease with fever, cough, and dyspnea (3). In Korea, the bark of the tree is also traditionally used to treat gout and neuralgia, and as an antipyretic agent (4). The tree has recently attracted considerable attention because of the broad range of pharmacological activities of its derivatives, including anti-diarrheal properties on ion transport of the rat intestinal epithelia (5-7). However, no data is currently available on the bioactive components, physiological properties, and hepatoprotective effects of ash tree leaves.

Acetaminophen (APAP, *n*-acetyl-*p*-aminophenol) is a commonly used analgesic and antipyretic drug at therapeutic doses, but overdoses can result in massive hepatic centrilobular necrosis and nephrotoxicity in both humans and experimental animals (7-9). APAP hepatotoxicity accounts for 20 to 75% of all cases of acute liver failure in the United States and Europe (10).

In an attempt to screen a new natural pharmaceutical material from ash tree leaves, we investigated the effect of the water extract obtained from these plant leaves on APAP-induced hepatotoxicity in mice. To determine the hepatoprotective effect of ash leaf extract, biochemical analysis of hepatospecific enzymes and histopathological observation of the liver were performed.

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Materials and Methods

The preparation of ash tree leaf extract Ash tree leaves were collected from the Ministry of Forestry in Daegu, Korea in May of 2004. About 2 kg of leaves were extracted using 20 L of water for 2 hr at 80°C. The extract was filtered and freeze-dried at -40°C. Dried ash tree leaf extract (ALE) was stored at 4°C until use. A voucher specimen was deposited in the Herbarium of the College of Medicine, Yeungnam University (Ash leaf 101).

Animal care and induction of liver damage by APAP Specific pathogen-free BALB/c mice (male, 6-8 weeks of age) were purchased from Hyochang Science Company (Umsung, Korea), and were housed in a temperature-controlled room under a constant 12-hr light and dark cycle. All of the animals were fed standard mouse chow (Sam Yang Food Co., Wonju, Korea) and had free access to water. The care of the animals was consistent with the National Institutes of Health guidelines on the care and use of laboratory animals. The mice were subjected to a 1-week adaptation period and were randomly divided into three groups of mice for the experiment: the normal control group (Normal), the APAP-treated group (APAP),

and the group pre-treated with ALE before treatment with APAP (ALE+APAP). In a preliminary study, APAP was administered at dosages of 300, 600, and 900 mg/kg body weight and pathologic examination of liver tissue was performed. Experimental liver damage was produced by the injection of APAP intraperitoneally at a dosage of 600 mg/kg body weight. This dose was sufficient to cause the development of evident liver injury, but was not severe enough to cause death. The ALE and APAP (Sigma Chemical Co., St. Louis, MO, USA) were dissolved in warm phosphate buffered saline (PBS). The ALE was administered orally at 300 mg/kg once daily for 7 consecutive days. On the 7th day, 3 hr after ALE treatment, mice were injected intraperitoneally with APAP solution. Animals in the normal group were given only PBS vehicle. Hepatotoxicity was determined 24 hr after APAP-induction and mice were fasted 15 hr before sacrificing. animals were anesthetized with sodium pentathol (40 mg/kg).

Biochemical assay Blood was collected from the abdominal aorta of each mouse with a heparinized syringe. The blood was centrifuged at 5,000×g for 5 min at 4°C to separate the plasma, and the hepatic enzyme activities were analyzed using a commercial kit (Asan Pharm. Co., Seoul, Korea).

Histological examinations Fresh liver tissue was fixed in 4% paraformaldehyde for 24 hr before routine histological processing. The hepatic tissue was subsequently dehydrated with a series of ethanol solutions from 75 to 100% before being embedded in paraffin wax. Cross-sections of a thickness of 5 μM were cut and stained with hematoxylin and eosin (H&E stain), or subjected to the terminal deoxynucleotidyl transferase-mediated dTUP nick end labeling (TUNEL staining) method. An apoptag apoptosis detection kit (Intergen Co., Purchase, NY, USA) was used according to the specifications of the manufacturer to selectively detect apoptotic cells in liver sections. The specimen was examined by light microscopy (Olympus Optical Co., Tokyo, Japan). For the Periodic acid-Schiff (PAS) stain, a 4 μM section of liver was immersed in a solution of 0.1% periodic acid for 15 min at 56°C. The slides were washed in running tap water and immersed in Schiff's reagent for 40 min. Sections were subsequently washed in running tap-water for 10 min, counter stained with Gill's hematoxylin, dehydrated in graded ethanol, cleared in xylene, and mounted in resinous medium. Stained sections were then observed by light microscopy. The histologic score was determined by diagnosis of a surgical pathologist according to inflammation, fatty change, degeneration of hepatocytes, necrosis, fibrosis, and regeneration:

(-) = none; no fibrosis
(+) = (mild) <33% of liver surface; mild portal fibrosis
(++) = (moderate) 33-66% of liver surface; periportal fibrosis
(+++) = (severe) >66% of liver surface; cirrosis

Statistical analysis of data All results were expressed as mean±standard error of mean. Statistical analyses were performed using an SPSS program. For multiple comparisons, a one way analysis of variance (ANOVA) and Duncan's multiple range tests were used. A p-value of <0.05 was considered significant.

Results and Discussion

APAP-induced hepatotoxicity in rodents is an animal model that is widely used to assess the hepatoprotective activities of new compounds (7, 11). At therapeutic doses, APAP is considered a safe drug. However, overdoses can cause hepatic necrosis, nephrotoxicity, extrahepatic lesions, and even death (12, 13). Overdoses of acetaminophen deplete glutathione stores (14), leading to the accumulation of NAPQI, mitochondrial dysfunction (13), acute hepatic necrosis (15), and apoptosis (9).

Plasma hepatic enzyme activities The effects of pretreatment with ALE on the APAP-induced elevation of plasma AST and ALT activities are shown in Table 1. A single dose of APAP (600 mg/kg) caused hepatotoxicity in BALB/c mice, as indicated by increases in plasma levels of AST and ALT. The increases were 3.1-fold for AST activity and 3.6-fold for ALT enzymes. However, at a dosage of 300 mg/kg of body weight for 7 days, oral ingestion of ALE by mice caused a decrease in the enzyme activity of AST by 44% and of ALT by 40%. The levels of hepatic enzymes such as AST and ALT primarily reflect the degree of liver damage and have been commonly used a diagnostic marker for hepatotoxicity (16). The rises in plasma AST and ALT levels have been attributed to the damaged structural integrity of the liver (2). This is because these enzymes are located in cytoplasm and are released into circulation after cellular damage (17). In this study, lower levels of plasma enzymes were observed from APAP-induced liver damage with ALE pretreatment. This may be due, in part, to the prevention of intracellular enzyme leakage by the membrane stabilizing activity of ALE. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes (18, 19). These effects of ash tree leaves reflect improvements in hepatocyte function.

Histological findings Table 2 shows a summary of the results of histological examination concerning the hepatoprotective effect of ALE pretreatment before APAP

Table 1. Effect of ash tree leaf extract on plasma enzyme activities in mice with APAP-induced heptotoxicity

Group[1)]	AST	ALT
	Karmen unit/L	
Normal	37.41±0.88[a,2)]	26.81±0.50[a]
APAP	116.46±2.30[c]	97.72±1.64[c]
ALE+APAP	76.94±2.44[b]	58.12±1.20[b]

[1)]Normal, Normal+PBS; APAP, APAP+PBS; ALE+APAP, Pretreated with ALE once daily for 7 days prior to APAP administration. Hepatotoxicity was determined 24 hr after APAP-induction.
[2)]Means±SE (n=7), The values with different superscripts were significantly different by Duncan's multiple range test (p<0.05).

Table 2. Effect of ash tree leaf extract on histopathology of mouse liver tissue with APAP-induced heptotoxicity

Group[1)]	Inflammation	Fatty change	Degeneration of hepatocytes	Necrosis	Regeneration	Fibrosis
Normal	–[2)]	–	–	–	–	–
APAP	++	–	+	+++	–	–
ALE +APAP	+	–	–	+	+	–

[1)]Normal, normal+PBS; APAP, APAP+PBS; ALE+APAP, group pretreated once daily with ALE for 7 days prior to APAP administration. Hepatotoxicity was determined 24 hr after APAP induction.
[2)]The histologic scores of inflammation, fatty change, degeneration of hepatocytes, necrosis, and regeneration used a system of pluses and minuses: -, none; +, mild change; ++, moderate change; +++, greatly affected.

injection. The normal group showed normal hepatic lobular architecture, portal tract, and central vein (Fig. 1A). The liver section treated with APAP alone showed moderate infiltration of inflammatory cells and severe centrilobular necrosis, which in some cases was also accompanied by congestion (Fig. 1B). In contrast, the extent of hepatic necrosis and inflammatory cell infiltration in the ALE-pretreated group was significantly lower than that in the APAP group (Fig. 1C). Additionally, mild regeneration was observed in the hepatic cells of mice pretreated with ALE before APAP injection. On the other hand, no hepatic fatty change or fibrosis were observed in either APAP-induced or ALE-pretreated groups. On TUNEL staining, large areas of TUNEL-positive hepatocytes were detected around the central veins in the APAP group, but pretreatment with ALE strongly attenuated the number of TUNEL-positive hepatocytes (Fig. 2C). Figure 3 shows light microscopic examination of cells highlighted by PAS stain for observation of glycogen content. In the normal group, the glycogen within the hepatocytes was stained red by PAS stain. The normal hepatocytes stained by PAS stain were red, which meant that high levels of glycogen were stored within hepatocytes. But APAP injection decreased hepatic glycogen content, which was observed as a pale red color upon PAS staining (Fig. 3B). In the group supplemented with ALE, the concentration of glycogen within the hepatocytes was greater than that of liver cells from rats treated with APAP alone (Fig. 3C). In our present study, histopathologic findings showed that APAP administration induced moderate inflammatory cell

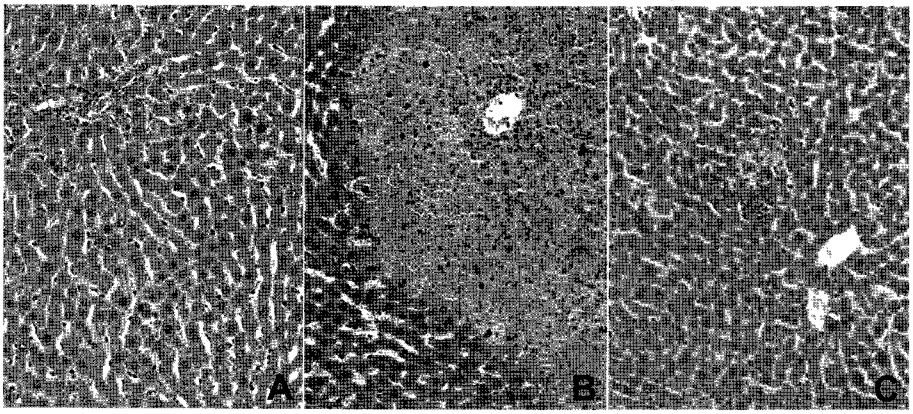

Fig. 1. Histologic findings of the liver (H&E stain, ×200). A, Normal group; B, APAP group; C, ALE+APAP group.

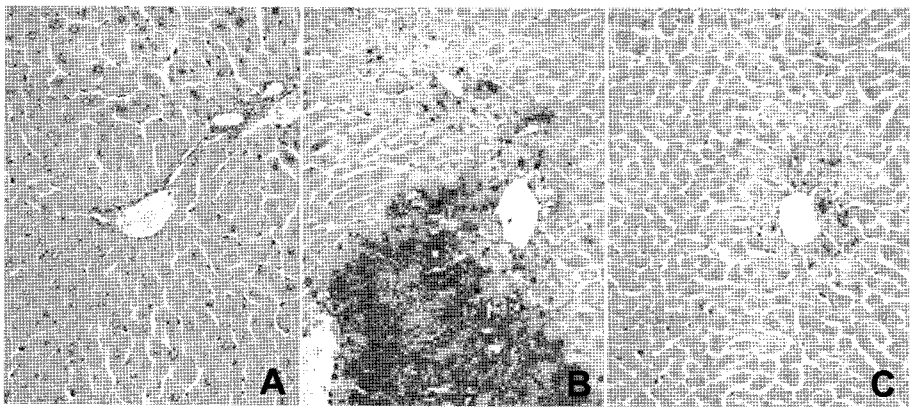

Fig. 2. Histologic findings in the liver from TUNEL staining (×200). A, Normal group; B, APAP group; C, ALE+APAP group.

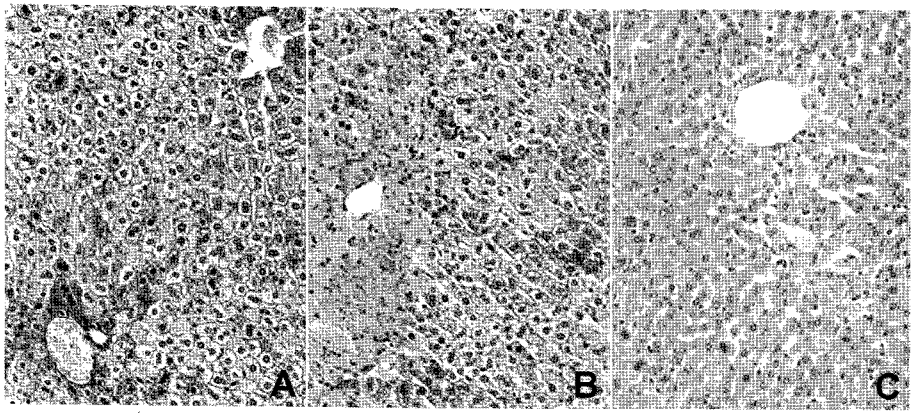

Fig. 3. Histologic findings for observation of glycogen content within the hepatocytes (PAS stain, ×200). A, Normal group; B, APAP group; C, ALE+APAP group.

infiltration, severe centrilobular necrosis as shown by H& E stain, and induced apoptosis as observed by microscopic examination of TUNEL-stained liver tissue (Table 2). However, hepatic damage and the number of TUNEL-positive hepatocytes were significantly alleviated by ALE pretreatment prior to APAP injection. Moreover, the morphology observed under PAS stain showed that ALE treatment helped prevent the decrease in hepatic glycogen content caused by APAP administration (Fig. 3C). This result means that ALE provides protection against hepatic necrosis, apoptosis, and other acute liver damage induced by APAP.

When we attempted to analyze the hepatoprotective components of ash tree leaves by GCMS, the main phenolic components were identified as esculetin, esculin, and fraxetin (data not shown). On the other hand, Kwon and Kim (4) reported that the chemical constituents of ash tree leaves were esculin, cichoriin, scopolin, and fraxin. Notably, the phenolic compound esculetin is known to exhibit a strong inhibitory effect on xanthine oxidase (20). It is interesting that the level of this enzyme was found to be high in subjects with hepatitis, mild hepatic intoxication, and brain tumors (20). Based on this observation, esculetin may act as an anti-hepatitis and/or anti-cancer agent.

In conclusion, extract of ash tree leaves protected against APAP-induced hepatotoxicity in mice as indicated by plasma levels of hepatic enzymes and histopathologic findings. These results support a medicinal use of these plant leaves against liver damage. Further study is necessary to establish which bioactive components isolated from these plant leaves has a hepatoprotective mechanism in an APAP-induced liver damage model.

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