The preventive effect of Daeshiho-tang on liver damage induced by acetaminophen in the rats.

Kim chang hyun, Kweon young mo, Lee young tae, Park sun dong
Department of Herbal pharmacology, Collage of Oriental Medicine, Dongguk University

국문초록

대시호탕이 acetaminophen으로 유도된 간독성 흰쥐에 미치는 영향

김창현, 권영모, 이영태, 박선동
동국대학교 한의과대학 방제학교실

Acetaminophen은 세계적으로 널리 사용되는 해열·진통제이지만, 또한 과용 및 남용, 알코올 중독과 같은 여러 원인에 의해 간독성을 유발할 수 있는 약물이다. 이러한 acetaminophen의 간독성은 CYP에 의해 생성되는 대사산물인 NAPQI와 활성산소에 의해 유발되는 것으로 알려져 있다.

본 연구에서 5주된 수컷 백서에 acetaminophen (500 ㎎/㎏)을 투여하기 전에 대시호탕 (500 ㎎/㎏)를 일주일간 투여하였다. 이 후 GOT, GPT, GST 그리고, 조직사진으로 대시호탕의 간보호작용을 측정하였다. 또한 대시호탕의 간보호작용 기전을 항산화작용과 CYP 2E1 발현조절을 통한 NAPQI 생성억제의 두 가지 면에서 측정하였다.

GOT, GPT 그리고 조직사진에서 나타난 결과들은 대시호탕이 고용량의 acetaminophen에 대한 간보호작용이 있음을 증명할 수 있었다. 또한 LPO와 catalase, 그

교신저자 : 박 선 동
경북 경주시 석장동 707 동국대학교 한의과대학 방제학교실
Tel : 054-770-2654, e-mail : sundong@dongguk.ac.kr]
접수 : 2004/ 12/ 21 채택 : 2004/ 12/ 27

리고 GSH 실험에서 나타난 결과들을 통해 대시호탕이 항산화작용이 있음을 알 수 있었다. 그리고, GSH, GST, RT-PCR, western blot 실험에서 대시호탕이 CYP 2E1의 발현을 조절하여 NAPQI 생성을 억제한다는 것도 알 수 있었다.

이상의 결과들을 바탕으로 대시호탕은 항산화작용에 의한 활성산소 제거력과 CYP 2E1의 발현조절을 통한 NAPQI 생성억제로 고용량 acetaminophen에 의해 유도된 간손상에 대해 유의성 있는 보호작용을 한다는 것을 알 수 있었다.

Key Word : 대시호탕, acetaminophen, CYP 2E1, NAPQI, 항산화

1. Introduction

Daeshiho-tang(DS) is a supplementary recipe of soshiho-tang and its source is synopsis of prescriptions of the golden chamber (金匱要略). The functions of DS are to treat soyang diseases and purge away internal stasis of heat. Patients with acute cholecystitis, cholelithiasis, acute pancreatitis and infection of abdominal activity can be treated by the modified recipe[1].

There are many drugs used to induce liver damage for the research of anti-hepatotoxicity effect. Among hepatotoxic animal models induced by drugs, acetaminophen(APAP) is one of these drugs. APAP is not only a drug for animal model but also a widely used analgesic and antipyretic drug throughout the world[2-3]. This is the reason why there are many researches about APAP hepatotoxicity and hepatoprotecitve drugs to prevent it. The hepatotoxicity of APAP is believed to be due to N-acetyl-p-benzoquinone imine(NAPQI) produced by CYPs and reactive oxygen species(ROS)[4].

Among these cytochrome p450(CYP)s, NAPQI which is mainly related to the hepatotoxicity of APAP is produced by four CYP forms: CYP 1A2, 2E, 3A and 2A5[5]. However, the experiments using CYP 2E1 knockout mice[6], CYP 1A2 2E1 double-null mice[7] and CYP 1A2 knockout mice[8] proved that CYP 2E1 has most important role in the hepatotoxicity of APAP.

In all types of liver damage, there is consistent evidence of enhanced production of free radicals and/or significant decrease of antioxidant defense. As a consequence, a large number of studies have focused on the pathogenetic significance of oxidative and nitrosative stress in liver injury as well as on therapeutic intervention with antioxidant and metabolic scavengers. Mitochondria and CYP enzymes are the main sources of ROS in hepatocytes acutely or chronically exposed to a "toxic" injury (environmental drugs, alcohol, therapeutical drugs, viruses, etc.). ROS also derive from Kupffer and inflammatory cells, in particular neutrophils. In hepatocytes, ROS may play a role in a very large cascade of reactions, such as Ca^{++} accumulation, circulatory status and transport function,

nitric oxide (NO) synthesis and metabolism, cytokine gene expression, caspase activity, growth factor synthesis and activity, DNA fragmentation, Na^+ influx, etc. Hence, a significant and sufficiently steady increase of ROS production leads to perturbation of the normal redox state and of the metabolism of the cell, with consequent impairment of various cell functions and activities, possibly until irreversible damage. ROS, in particular superoxide anion, rapidly react with NO to form peroxynitrite, that may act as a dangerous molecule by influencing some SH-related enzyme activities and by cooperating to induce membrane lipid peroxidation. By inducing glutathione(GSH) depletion, ROS induce oxidative stress and reduce the antioxidant capability of other antioxidants. Depletion of GSH may also be a consequence of liver damage. Indeed, a large literature documented the decrease of GSH in liver and circulation in patients with alcoholic and viral cirrhosis, and, more recently, in those with HCV-related chronic hepatitis. In this last group the bioavailability of GSH influences both the entity of liver apoptosis and necrosis and the response to antiviral therapies[9].

It is reported that DS has a hepatoprotective effect[1], but there are a few reports about its mechanism, especially related to the regulation of CYP. Therefore I intended to demonstrate the mechanism of DS's hepatopreventive effect on liver injury induced by APAP with two approaches; antioxidant effect and inhibition of NAPQI production. I investigated the DS's antioxidant effect and regulatory effect on the expression of CYP 2E1 in the rat. My findings demonstrate that DS has anti-hapatotoxicity effect against overdose APAP, which originates from its antioxidant effect and reducible effect of NAPQI production through the regulation of CYP 2E1 expression.

2. Materials and methods

1. Plant material (Daeshiho-tang extraction)

DS contents for this study were purchased from Dongguk University Oriental Medical Hospital.

DS extraction was prepared as follows: 80% methanol which is 3 times as much as DS was added to DS 200 g and maintained at room temperature for 48 h. The residues were removed by filtration 2 times and the filtrate was evaporated to obtain the desired concentration (2 g dry plants equivalent extract/㎖). The liquid was centrifuged at 7,000 rpm for 30 min, and the supernatant was lypophilized by freeze-drier to give a powder (yield, 2.1%). The dried extract was resolved in distilled water to appropriate concentrations.

Table 1. Contents and amount of Daeshiho -tang(DS)

韓藥名	Drug name	Amount
柴 胡	*BUPLEURI RADIX*	16 g
黃 芩	*SCUTELLARIAE RADIX*	10 g
大 黃	*RHEI RADIX ET RHIZOMA*	8 g
枳 實	*AURANTII IMMATURUS FRUCTUS*	6 g
半 夏	*PINELLIAE RHIZOMA*	4 g
芍 藥	*PAEONIAE RADIX ALBA*	10 g
生 薑	*ZINGIBERIS RHIZOMA RECENS*	4 g
大 棗	*JUJUBAE FRUCTUS*	2 g
Total amounts		60 g

2. Animals and treatments

Male sprague dawley rats (5 weeks old) were purchased from Korea Hyochang Science Co. and housed ten per cage in plastic cages. The animals were maintained on a 12h light/dark cycle under controlled temperature (23±3 ℃) and humidity (55±5%) for 1 week before experimental use. They were allowed free access to standard laboratory food and water, but were fasted the night before the experiment. During food deprivation and experimentation, they were housed in cages with wire-mesh floors to prevent them ingesting their bedding

Material and excreta.

DS at 500 ㎎/㎏ (p.o.) was administered to the rats for one week, and then APAP at 500 ㎎/㎏ (abdominal injection) was given 24h after DS administration. APAP was administered between 9 and 11 a.m. to avoid any circadian variation. DS and APAP were suspended in distilled water and DMSO, respectively. The dose volume of each compound and vehicle combined was 10 ㎖/㎏. Rats in the 'vehicle' group (control) were fasted and given APAP but not DS; instead of DS they received only distilled water.

The rats were anesthetized with diethyl ether and blood samples were taken from the right ventricle with a heparinized syringe and then their livers were removed 24h after APAP administration. The livers were frozen immediately and stored in - 70 ℃ deep freezer until assayed.

3. Chemicals

APAP, bovine serum albumine, 5,5'-dithio-bis-(2-nirtiobenzoic acid) (DTNB), sodium dodecyl sulfate (SDS), thiobarbituric acid (TBA) were purchased from Sigma (St. Louis, MO, USA).

Glutamate oxaloacetate transaminase (GOT) and glutamate pyruvate transaminase (GPT) kits were purchased from Asan pharmaceutical Co. (Seoul, Korea).

Ultra Clean Tissue RNA Isolation Kit. was purchased from MO BIO Laboratories Inc (Solana Beach, CA, USA).

CYP 2E1 primer and β-actin primer was purchased from Corebio Co. (Seoul, Korea).

Rabbit anti-human/rat CYP 2E1 antibody and anti-rabbit IgG was purchased from Chemicon Co (CA, USA) and GAPDH antibody and anti-goat IgG was purchased from Santacruz Co (USA).

Enhanced chemiluminoesence (ECL) solution was purchased from Amersham Co. (USA) and X-ray film from Kodak Co. (Japan).

All the other chemicals used were of analytical grade.

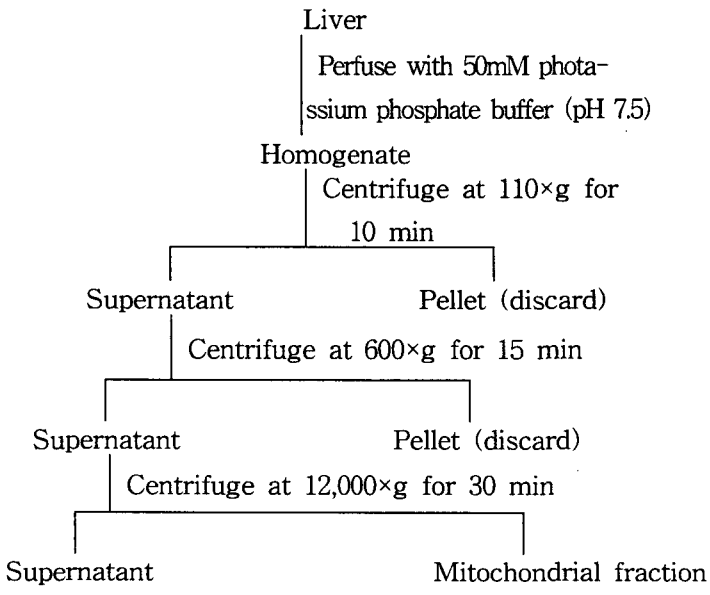
4. Histopathological examination

Rats were allowed free access to food 9h after APAP administration, and survival was monitored until 48h after the APAP had been given. Rats surviving 48h after APAP administration were anesthetized with diethyl ether and their livers were removed for histopathological examination. The livers were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The extent of liver necrosis was quantitated by the point-counting method (Mori et al., 1980). To estimate the extent of necrosis, the specimen was observed under a light microscope with an eyepiece grid (441 points/㎠). The extent of necrosis was expressed as the percentage of test points lying on the profile of the necrotic parenchyma within the fields observed.

5. Mitochondrial fraction

The liver was homogenized by 50 mM potassium phosphate buffer (pH 7.5) in homogenizer for 4 minutes. After the homogenate was centrifuged at 110×g for 15 min, some of the supernatant was used for LPO assays and GSH assays and the other was centrifuged again at 600×g for 15 minutes. The pellet was removed and the supernatant was centrifuged again at 12,000×g for 30 minutes. After centrifugation, the supernatant was used for catalase assays and GST assays(scheme 1.).

Scheme 1. Preparation of hepatic mitochondrial fractions for enzyme studies



6. GOT, GPT assays

GOT and GPT was measured with a spectrophotometric diagnostic kit obtainded from the Asan pharmaceutical Co.(Korea). The absorbance at 505 nm was read and results are expressed as karmen unit/㎖.

7. Hepatic lipid peroxidation (LPO) assays

Hepatic LPO was measured by the formation of the thiobarbituric acid-reactive material, malondialehyde (MDA). Briefly, after the homogenate was centrifuged at 1,000×g, the supernatant was mixed with 8.1% sodium dodecyl sulfate, 20% acetate buffer (pH 3.5) and 0.8% thiobarbituric acid (TBA) and maintained at 95℃ for 1 hour. Cooled up to room temperature, producted TBA reactive substance was added to n-utanol and pyridine mixture (15:1). The absorbance at 532 nm was read and results are expressed as nmole/mg.

8. Glutathione(GSH) assays

GSH were determined as follow.

As mentioned above, the homogenate for GSH assays was centrifuged 1,000×g and 4% sulfosalicylic acid added to the supernatant obtained by centrifugation. After centrifugation at 1,000×g for 10 minutes, the supernatant was mixed with 1 mM DTNB and maintained at room temperature for 20 minutes and the absorbance at 412 nm was read. Results are expressed as nmole/㎎.

9. Glutathione-s-transrerase(GST) assays

GST were determined by using chiorodinitro-benzene(CDNB) and GSH. After the homogenate for GST assays was diluted with 0.1 M potassium phosphate buffer(pH 6.5), 1 mM GSH and 1 mM CDNB were added to it. The CDNB conjugated for 1 minute was measured spectrophotometrically at 340 nm and the results are expressed as nmole.

10. Catalase assays

Catalase was assayed at 20 ℃. Previously, liver homogenates aliquots were centrifuged at 1,000×g for 10 min and the supernatants were used for the enzymatic assay. The reaction mixture contained 0.1 mM EDTA, 10 mM H_2O_2, 0.002% Triton X-100, a suitable amount of protein and 0.5 M potassium phosphate buffer, pH 7.0, in a final volume of 1 ㎖. The absorbance at 240 nm was read. The enzymatic activity is expressed as unit which means enzyme contents to decompose 1 micromole (μmol) H_2O_2 for 1 minute.

11. Western blot analysis

Frozen liver tissue (about 100 ㎎) was homogenized in 1.5 ㎖ of sample buffer [50 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10 mM dithiothreitol, 10% glycerol and 1 mM phenylmethylsulfonyl fluoride], boiled for 5 min, passed through a 22-gauge needle three times to shear the DNA and stored at -70 °C until use. The protein content of each sample was determined and adjusted to a concentration of 2.5 mg/ml, and

bromophenol blue was added to each sample to a final concentration of 0.01%. The samples were boiled again for 5 min, then 50g of protein from each sample was separated by SDS polyacrylamide gel electrophoresis with 12.5% polyacrylamide gel and electroblotted on to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). After non-specific binding sites had been blocked in blocking buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl and 5% skim milk) for 18h, the PVDF membrane was incubated for 2h with mouse anti-CYP 2E1 antibody at room temperature with occasional shaking. The antibody was used at a 1:1000 dilution in washing buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl and 0.05% Tween 20) containing 0.5% BSA. It was then washed four times with washing buffer, incubated for a further hour at room temperature (with occasional shaking) with horseradish peroxidase-conjugated goat anti-rabbit antibody (Amersham Pharmacia Biotech, Buckinghamshire, England) at a 1:3000 dilution in washing buffer containing 0.5% BSA, and then washed four times with washing buffer. The immunoblot was revealed with an ECLTM Western blotting analysis system (Amersham Pharmacia Biotech). The PVDF membrane was exposed to Hyperfilm-ECL (Amersham Pharmacia Biotech, Buckinghamshire, England) to localize antibody binding.

12. Reverse transcriptase-polymerase chain reaction(RT-PCR)

Total RNA was isolated from frozen liver samples using an UltraClean Tissue RNA Isolation Kit(MO BIO Laboratories Inc, Solana Beach, CA, USA).

First strand cDNA was synthesised using 1 μg total RNA from lymphocytes and 200 U MMLV reverse transcriptase, using 500ng oligo dT as a primer. The reaction was carried out at 37℃ for 1h. PCR conditions for CYP 2E1 were: 1 $\mu\ell$ cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 80M each for dATP, dGTP, dTTP and dCTP; 20 pmol of each primer, 2.5 U Taq polymerase, and 30 cycles (94 ℃, 1 min; 60 ℃, 1 min; 72 ℃, 1 min). Specific CYP 2E1 primers were designed according to the sequence: 5′-GAAAAAGCCAAGGAAC ACC-3′ (sense) and 5′-GCAGACAGGAGCAGAAACA-3′ (antisense). PCR products were analysed in 2% agarose gels.

For semi-quantitative RT-PCR assays, a co-amplification approach was used with β-actin as the control gene. Rat liver and lymphocyte cDNA was synthesised from 4g total RNA. The PCR conditions were the same as described above, except that 10 pmol β-actin primers (sense: 5′-CAGCCTTCCTTCCTGGG TATG-3′ and antisense: 5′-TAGAGCCACCAATCCACACAG-3′) were also used. The PCR products were analysed after ethidium SHomide staining of agarose gels and the ratio between CPY 2E1 and β-actin was determined by densitometry.

13. Protein assay

Protein contents were determined by the method of Lowry et al.[10], with bovine serum albumin as a standard.

14. Densitometric and statistical analysis

The intensity of the bands from western blot analysis and RT-PCR was estimated with Gel-Print System (Core Bio Corp., Seoul, KOREA). The values are expressed as means±SE. Statistical analyses were performed with Sigma Plot 2001(window, version 7.0). Statistical analysis

3. Result

1. The effect of DS on serum GOT levels

To investigate the hepatoprotective effect of DS, serum GOT levels were measured after APAP treatment of rats for 24h. The results are as follow: normal 23.87±4.11 karmen unit/㎖, control 36.03±5.83 karmen unit/㎖, DS 29.97±3.69 karmen unit/㎖. As indicated in the graph, GOT level of control group were significantly increased compared with normal group ($p<0.01$), but those of SH group were decreased compared with control group ($p<0.05$)(Fig. 1).

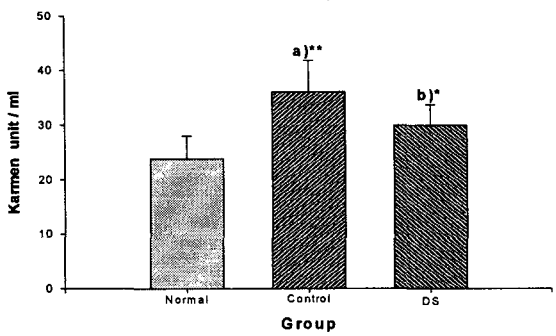


Figure 1. The effect of DS on serum GOT levels with hepatic damage induced by APAP (APAP). Each group was treated with DMSO (normal), 500 ㎎/㎏ APAP dissolved in DMSO (control), 500 ㎎/㎏ APAP dissolved in DMSO after administration of 500 ㎎/㎏ DS dissolved in distilled water for 7 days(DS), respectively.

** : $p < 0.01$, * : $p < 0.05$

a) : compared with normal group.

b) : compared with APAP group.

2. The effect of DS on serum GPT levels

To know the hepatoprotective effect of DS, serum GPT levels were also measured after APAP treatment of rats for 24h. The results are as follow: normal 68.37±9.86 karmen unit/㎖, control 153.55±17.21 karmen unit/㎖, DS 136.66±13.27 karmen unit/㎖. As indicated in the graph, GPT level of control group were significantly increased compared with normal group ($p<0.01$), but those of DS group were decreased compared with control group ($p<0.05$)(Fig. 2).

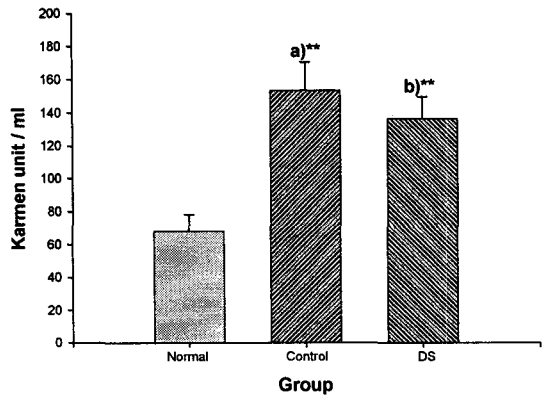


Figure 2. The effect of DS on serum GPT levels with hepatic damage induced by APAP (APAP). Each group was treated with DMSO (normal), 500 ㎎/㎏ APAP dissolved in DMSO (control), 500 ㎎/㎏ APAP dissolved in DMSO after administration of 500 ㎎/㎏ DS dissolved in distilled water for 7 days(DS), respectively.

** : $p < 0.01$,

a) : compared with normal group.

b) : compared with APAP group.

3. Light micrograph of paraffin -embedded rat liver

Histological examination(Fig. 3) showed that APAP induced the typical changes of confluent centrilobular necrosis compared with control (normal group). This necrosis was attenuated with the administration of DS.

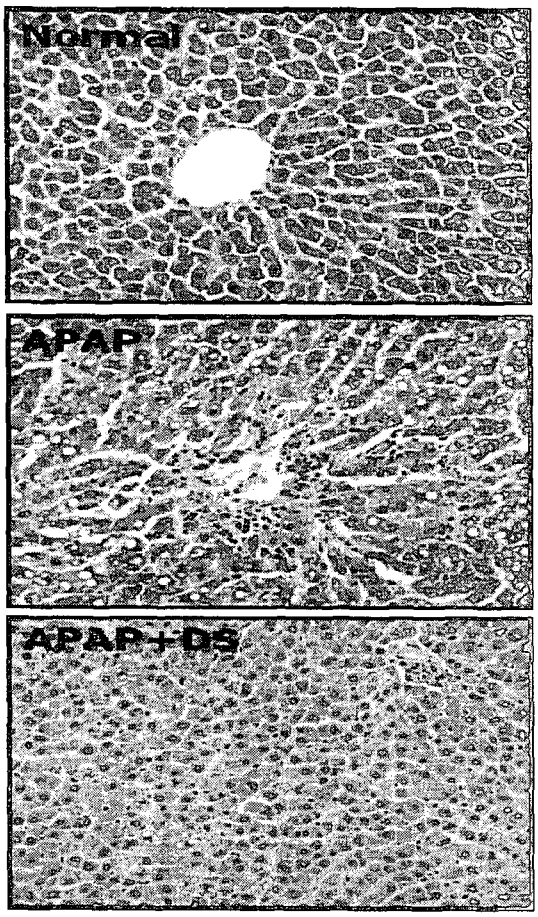


Figure 3. Light micrograph of paraffin-embedded rat liver. All sections were stained with hematoxylin and eosin.

(Control) Photomicrograph (×200) of a section taken from the liver of normal control group rat treated with DMSO.

(APAP) Photomicrograph (×200) of a section taken from the liver of APAP group rat treated with 500 ㎎/㎏ APAP dissolved in DMSO.

(APAP+DS) Photomicrograph (×200) of a section taken from the liver of DS group rat treated with 500 ㎎/㎏ APAP dissolved in DMSO after administration of 500 ㎎/㎏ DS dissolved in distilled water for 7 days.

4. The effect of DS on lipid peroxidation(LPO) levels

The formation of lipid peroxidation indicates cellular injury mediated by reactive oxygen intermediates with resultant destruction of membrane lipids and production of lipid peroxides. This study showed that APAP enhanced LPO levels significantly (28.41±3.41 nmole/㎖ ; $p<0.01$) compared with normal group (11.57±1.65 nmole/㎖), but DS decreased it compared with control group (24.96±2.78 nmole/㎖ ; $p<0.05$)(Fig. 4).

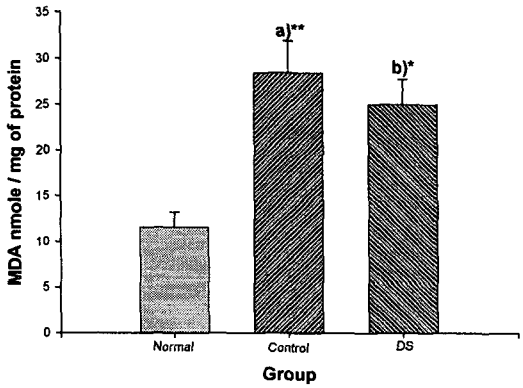


Figure 4. The effect of DS on lipid peroxidation (LPO) levels with hepatic damage induced by APAP (APAP). Each group was treated with DMSO (normal), 500 ㎎/㎏ APAP dissolved in DMSO (control), 500 ㎎/㎏ APAP dissolved in DMSO after administration of 500 ㎎/㎏ DS dissolved in distilled water for 7 days(DS) respectively.

** : $p < 0.01$, * : $p < 0.05$

a) : compared with normal group.

b) : compared with APAP group.

5. The effect of DS on catalase activity

To assess the defense mechanisms involved in the APAP-induced oxidative stress, the activity of catalase was determined. The data showed that APAP potently decreased catalase activity (6.21±0.96 nmole/㎖; $p<0.01$) compared with normal group (10.37±1.02nmole/㎖). In the DS group, the catalase activity were increased (7.77±1.32 nmole/㎖ ; $p<0.05$) compared with control group(Fig. 5).

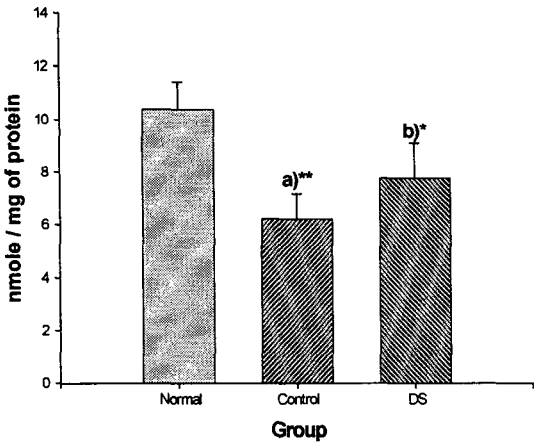


Figure 5. The effect of DS on catalase activity with hepatic damage induced by APAP (APAP). Each group was treated with DMSO (normal), 500 ㎎/㎏ APAP dissolved in DMSO (control), 500 ㎎/㎏ APAP dissolved in DMSO after administration of 500 ㎎/㎏ DS dissolved in distilled water for 7 days (DS) respectively.

** : $p < 0.01$, * : $p < 0.05$

a) : compared with normal group.

b) : compared with APAP group.

6. The effect of DS on glutathione (GSH) contents

As GSH plays an important role in the detoxification of APAP, the hepatic GSH were examined 24h after the administration of APAP. The hepatic GSH of the control group were significantly decreased (15.55±0.94 nmole/㎖ ; $p<0.01$) compared with noraml group (18.68±1.90 nmole/㎖), where as those of DS group were significantly increased (17.08±1.11 nmole/㎖ ; $p<0.01$) compared with control group. But the hepatic GSH of DS group did not reach the initial level (Fig. 6).

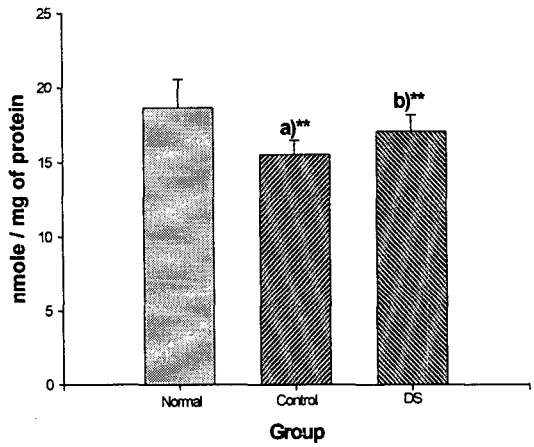


Figure 6. The effect of DS on glutathione (GSH) contents with hepatic damage induced by APAP (APAP). Each group was treated with DMSO (normal), 500 ㎎/㎏ APAP dissolved in DMSO (control), 500 ㎎/㎏ APAP dissolved in DMSO after administration of 500 ㎎/㎏ DS dissolved in distilled water for 7 days (DS) respectively.

** : $p < 0.01$

a) : compared with normal group.

b) : compared with APAP group.

7. The effect of DS on glutathione-s-transferase(GST) levels

As the function of GST is the conjugation between GSH and NAPQI(APAP toxic metabolite), the hepatic GST levels were examined 24h

after the administration of APAP. The hepatic GST levels of the control group were significantly enhanced (3.74±0.44 nmole/㎖ ; $p<0.01$) compared with noraml group (2.51±0.14 nmole/㎖). But, those of DS group were decreased (3.24±0.33 nmole/㎖ ; $p<0.05$) compared with control group(Fig. 7).

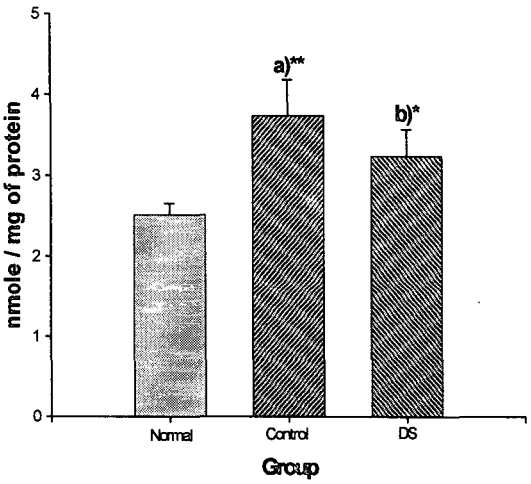


Figure 7. The effect of DS on glutathione-s -transferase (GST) levels with hepatic damage induced by APAP (APAP). Each group was treated with DMSO (normal), 500 ㎎/㎏ APAP dissolved in DMSO (control), 500 ㎎/㎏ APAP dissolved in DMSO after administration of 500 ㎎/㎏ DS dissolved in distilled water for 7 days (DS), respectively.
** : $p < 0.01$, * : $p < 0.05$
a) : compared with normal group.
b) : compared with APAP group.

8. The effect of DS on the CYP 2E1 mRNA expression levels

To examine the involvement of DS on the CYP 2E1 mRNA expression levels, RT-PCR was used. As shown in the Fig. 8, APAP significantly increased the expression of CYP 2E1 mRNA compared with Normal group, but the administration of DS prevented the expression of CYP 2E1 mRNA induced by APAP like the lane 2.

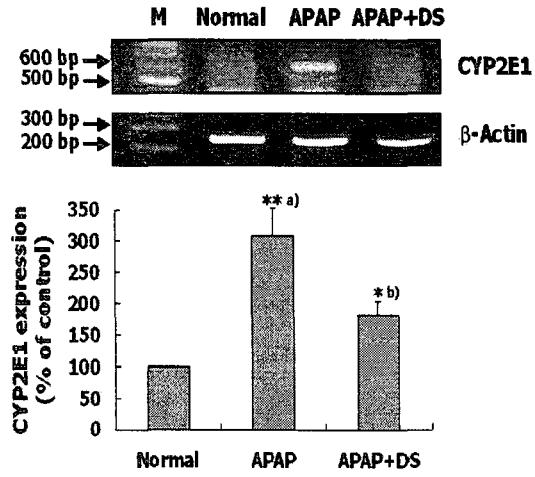


Figure 8. The effect of DS on the CYP 2E1 mRNA expression levels with the rat liver treated with DMSO (normal, lane 1), 500 ㎎/㎏ APAP dissolved in DMSO (APAP, lane 2), 500 ㎎/㎏ APAP dissolved in DMSO after administration of 500 ㎎/㎏ DS dissolved in distilled water for 7 days (APAP+DS, lane 3), respectively. Rat CYP 2E1 (517 bp) PT-PCR prducts were loaded in 2% ETDS-stained agarose gel.
** : $p < 0.01$, * : $p < 0.05$
a) : compared with normal group.
b) : compared with APAP group.

9. The effect of DS on the CYP 2E1 protein expression levels

To examine the involvement of DS on the CYP 2E1 protein expression levels, as a method to confirm the result of RT-PCR, western blot analysis was used. As shown in the Fig 9, APAP significantly increased the expression of CYP 2E1 protein compared, with normal group, but the administration of DS decreased the expression of CYP 2E1 protein induced by APAP like the lane 2. These results were

consistent with those of Fig. 8(mRNA).

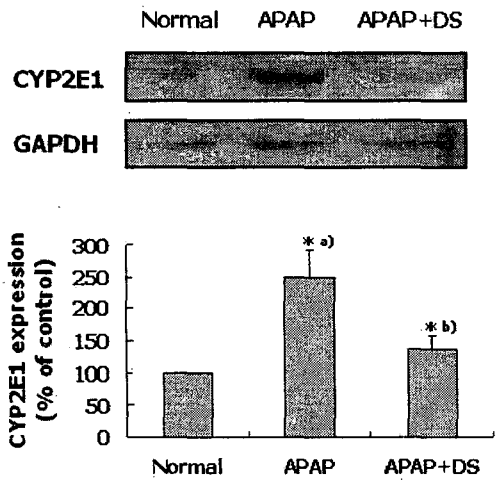


Figure 9. The effect of DS on the CYP 2E1 protein expression levels with the rat livers treated with DMSO (normal, lane 1), 500 mg/kg APAP dissolved in DMSO (APAP, lane 2), 500 mg/kg APAP dissolved in DMSO after administration of 500 mg/kg DS dissolved in distilled water for 7 days (APAP+DS, lane 3), respectively. The samples of the SDS-PAGE were total proteins from the rat livers of each group. Western blot was carried out by using rat CYP 2E1 specific polyclonal antibody and being loaded in 10% SDS-PAGE gel.

** : $p < 0.01$, * : $p < 0.05$

a) : compared with normal group.

b) : compared with APAP group.

4. Discussion

The liver is the largest organ in the body, contributing about 1/50 of the total body weight or about 1.5kg in the average adult human. The basic functional unit of the liver is the liver lobule, which is a cylindrical structure several millimeters in length and 0.8 to 2 millimeters in diameter. The liver performs many different functions. Yet the liver is also a discrete organ, and many of its functions interrelate with one another. This becomes especially evident in abnormalities of the liver, because many of its functions are disturbed simultaneously. The liver's different functions include (1) filtration and storage of blood, (2) metabolism of carbohydrates, proteins, fats, hormones, and foreign chemicals, (3) formation of bile, (4) storage of vitamins and iron, and (5) formation of coagulation factors[11].

Drugs continue to be pulled from the market with disturbing regularity because of late discovery of hepatotoxicity. Such unexpected toxicities appear to be the consequence of the unique vascular, secretory, synthetic, and metabolic features of the liver. About 75% of hepatic blood comes directly from the gastrointestinal viscera and spleen via the portal vein. Portal blood brings drugs and xenobiotics absorbed by the gut directly to the liver in concentrated form. Drug-metabolizing enzymes such as cytochrome p450 (CYP) detoxify many xenobiotics but activate the toxicity of others. The xenobiotics which are activated by drug-metabolizing enzymes include APAP, CCl_4 and so forth[12].

APAP overdose causes acute liver injury in both humans and animals. APAP overdose results in the accumulation of a highly reactive and cytotoxic intermediate, NAPQI, which may cause centrilobular necrosis of hepatocytes. Hepatocyte apoptosis has also been shown to be a significant contributor to APAP-induced liver injury and to precede necrosis. Reactive oxygen species, nitric oxide, lipid peroxidation, and disordered calcium

homeostasis are mechanisms that may have a contributory role in the development of liver injury after APAP excess[13].

In this study, in the beginning we examined not only serum GOT and GPT, to prove the hepatoprotecitive effect of DS, but also histological examination[14].

Then, I investigated the mechanism of its hepatoprotective effect by two approaches; the antioxidant effect on ROS and the inhibitory effect on NAPQI production by regulating the expression of CYP 2E1 known as main producer of NAPQI. The antidotal mechanisms against overdose APAP are divided into four types; Cysteine prodrug related with GSH, CYP inhibitor, conjugation enhancer related with glucuronic acid and antioxidant[15]. Based on these, first, I examined LPO and catalase activity which are the detoxification mechanism related with antioxidant effect[16-17].

Second, I confirmed GSH and GST which are the potential indirect indicator of NAPQI production as well as the detoxification mechanism related with conjugation of NAPQI[18].

Finally, I checked the expression of CYP 2E1 mRNA and protein using RT-PCR and western blot analysis, which are the detoxification mechanism related with CYP inhibitor[19].

GOT and GPT assay were used to check the liver damage. GOT and GPT assay showed that GOT and GPT levels of DS group, to which DS were administered before APAP injection, were decreased compared with those of control group increased by APAP (Fig. 1, Fig. 2). In addition, histological examination(Fig. 3) showed that APAP-induced necrosis was attenuated with the administration of DS. Based on these data, I concluded that DS has a hepatoprotective effect against overdose APAP.

To assess the defense mechanisms involved in the APAP-induced oxidative stress, LPO levels and catalase activity were examined. This study showed that the admistration of DS decreased LPO levels compared with APAP-injected group(Fig. 4), and in the DS group, the catalase activity were increased compared with control group (Fig. 5). These results indicate that antioxidant function of DS is involved in the mechanism of its hepatoprotective effect on the liver damage induced by overdose APAP.

The conjugation between GSH and NAPQI, which may be metabolized in part by GST, plays an important role in the detoxification of APAP, and the reduction of both GST and GSH may be a maker to indicate the decline of NAPQI production indirectly. The hepatic GSH and GST were examined 24h after the administration of APAP. The hepatic GSH of the DS group were increased compared with control group (Fig 6.) and the hepatic GST levels of DS group were decreased compared with control group(Fig 7.). These results suggest indirectly that the administration of DS reduces the production of NAPQI. Besides, because GSH is the defence system for not only NAPQI but also ROS and hepatic GST is a sensitive indicator of hepatocelluar injury, the result from GSH (Fig. 6) also demonstrate that DS has the

antioxidant function, and the decrease of GST in the DS group, compared with Control group (Fig 7.), shows that DS diminished the liver injury induced by APAP.

To further study and confirm the results of GST and GSH assay, I check the expression of CYP 2E1 mRNA and protein using RT-PCR and western blot analysis. This is because CYP 2E1 is reported to be a main producer of NAPQI and suppression the expression of CYP 2E1 means directly the reduction of NAPQI production. As shown in the Fig. 8, APAP significantly increased the expression of CYP 2E1 mRNA compared with normal group, but the administration of DS suppressed the expression of CYP 2E1 mRNA. Also, shown in the Fig. 9, the result of western blot analysis was consistent with those of RT-PCR. These results made us conclude that the administration of DS has the regulatory effect on the expression of CYP 2E1.

Taken together, the results of the present study demonstrate that DS treatment before overdose APAP injection, can protect rats against APAP-induced hepatotoxicity. The data taken from GOT, GPT, histological examination and GST indicated that DS has a significant antidotal effect on APAP overdose. Also, the date taken from LPO and catalase showed that DS has a antioxidant effect which is one of mechanisms of the hepatoprotective effect of DS. Finally, the data including GSH, GST, RT-PCR, Western blot demonstrated that DS has a suppressive effect on the production of NAPQI, by regulating the expression of CYP 2E1. Therefore, I concluded that the mechanisms underlying the protective effect of DS against APAP-induced liver damage are both the inhibitory effect on the expression of CYP 2E1, which leads to the suppression of NAPQI production, and the antioxidant effect against ROS.

5. Conclusion

Daeshiho-tang(DS) is a supplementary recipe of Soshiho-tang and its source is Synopsis of prescriptions of the golden chamber(金匱要略). According to reports, it is said that DS has a hepatoprotective effect, but there are no report about its mechanism related to the regulatory effect on CYP 2E1 expression and antioxidants effect on ROS, both of which have been known as two toxins connected with APAP-induced hepatotoxicity. Therefore we intended to demonstrate the mechanism of DS's hepatopreventive effect on liver injury induced by APAP with two approaches; the antioxidant effect on ROS and the inhibitory effect on NAPQI production by regulating the expression of CYP 2E1 known as the most important enzyme for NAPQI.

In this study, male sprague dawley rats (5 weeks old) were administrated DS (500 ㎎/㎏) for one week before APAP (500 ㎎ /㎏) injected.

The data including GOT, GPT, histological examination and GST indicated that DS has a significant antidotal effect

on APAP overdose. Also, the date including LPO and catalase showed that DS has a antioxidant effect which is one of mechanisms of the hepatoprotective effect of DS. Finally, the data including GSH, GST, RT-PCR, Western blot showed that DS has a suppressive effect on the production of NAPQI, by regulating the expression of CYP 2E1.

Based on these data, I concluded that the mechanisms underlying the protective effect of DS against APAP-induced liver damage are both the antioxidant effect against ROS and the inhibitory effect on the expression of CYP 2E1, which leads to the suppression of NAPQI production.

References

1. Zhang Enqin et al. Prescriptions of traditional chinese medicine, Shanghai, Shanghai medical college press, 1988.
2. Ramaiah SK, Apte U, Mehendale HM. Cytochrome P4502E1 induction increases thioacetamide liver injury in diet-restricted rats. Drug Metab Dispos. 2001 Aug;29(8):1088-95.
3. Barkin RL. APAP, aspirin, or Ibuprofen in combination analgesic products. Am J Ther., 2001 Nov-Dec;8(6):433-42.
4. James LP, Mayeux PR, Hinson JA. APAP-induced hepatotoxicity. Drug Metab Dispos. 2003 Dec;31(12):1499-506.
5. DiPetrillo K, Wood S, Kostrubsky V, Chatfield K, Bement J, Wrighton S, Jeffery E, Sinclair P, Sinclair J. Effect of caffeine on APAP hepatotoxicity in cultured hepatocytes treated with ethanol and isopentanol. Toxicol Appl Pharmacol. 2002 Dec 1;185(2):91-7.
6. Lee SS, Buters JT, Pineau T, Fernandez-Salguero P, Gonzalez FJ. Role of CYP2E1 in the hepatotoxicity of APAP. J Biol Chem. 1996 May 17;271(20):12063-7.
7. Zaher H, Buters JT, Ward JM, Bruno MK, Lucas AM, Stern ST, Cohen SD, Gonzalez FJ. Protection against APAP toxicity in CYP1A2 and CYP2E1 double-null mice. Toxicol Appl Pharmacol. 1998 Sep;152(1):193-9.
8. Tonge RP, Kelly EJ, Bruschi SA, Kalhorn T, Eaton DL, Nebert DW, Nelson SD. Role of CYP1A2 in the hepatotoxicity of APAP: investigations using Cyp1a2 null mice. Toxicol Appl Pharmacol. 1998 Nov;153(1):102-8.
9. Loguercio C, Federico A. Oxidative stress in viral and alcoholic hepatitis. Free Radic Biol Med. 2003 Jan 1;34(1):1-10.
10. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951 Nov;193(1):265-75.
11. Arthur C. Guyton et al. Medical Physilogy 10/e, Philadelphia, W.B. Saunders, 2000.
12. Caro AA, Cederbaum AI. Oxidative stress, toxicology, and pharmacology of CYP2E1. Annu Rev Pharmacol Toxicol. 2004;44:27-42.
13. Waters E, Wang JH, Redmond HP, Wu QD, Kay E, Bouchier-Hayes D. Role of taurine in preventing APAP -induced hepatic injury in the rat. Am J Physiol Gastrointest Liver Physiol.

2001 Jun;280(6):G1274-9.
14. Lin CC, Chang CH, Yang JJ, Namba T, Hattori M. Hepatoprotective effects of emodin from Ventilago leiocarpa. J Ethnopharmacol. 1996 Jun;52(2):107-11.
15. Sumioka I, Matsura T, Yamada K. Therapeutic effect of S-allylmercapto-cysteine on APAP-induced liver injury in mice. Eur J Pharmacol. 2001 Dec 21;433(2-3):177-85.
16. Choi D, Leininger-Muller B, Kim YC, Leroy P, Siest G, Wellman M. Differential role of CYP2E1 binders and isoniazid on CYP2E1 protein modification in NADPH-dependent microsomal oxidative reactions: free radical scavenging ability of isoniazid. Free Radic Res. 2002 Aug;36(8):893-903.
17. Jeon TI, Hwang SG, Lim BO, Park DK. Extracts of Phellinus linteus grown on germinated brown rice suppress liver damage induced by carbon tetrachloride in rats. Biotechnol Lett. 2003 Dec;25(24):2093-6.
18. Kozer E, Evans S, Barr J, Greenberg R, Soriano I, Bulkowstein M, Petrov I, Chen-Levi Z, Barzilay B, Berkovitch M. Glutathione, glutathione-dependent enzymes and antioxidant status in erythrocytes from children treated with high-dose paracetamol. Br J Clin Pharmacol. 2003 Mar;55(3):234-40.
19. Jeong HG. Inhibition of cytochrome P450 2E1 expression by oleanolic acid: hepatoprotective effects against carbon tetrachloride-induced hepatic injury. Toxicol Lett. 1999 Apr 12;105(3):215-22.