

Effects of *Galhwahyejung-tang* (GHT) on Protection for Alcohol-induced Liver Injury

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Objective : The purpose of this study was to examine the protective efficacy of GHT on alcoholic liver injury.

Methods : We measured the rate of alcohol oxidation, serum level of liver enzyme, lipid peroxidation level in liver tissue, and inflammatory related cytokine expressions in the liver.

Results : GHT showed liver protective effects, lowered the levels of AST and LDH in serum and inhibited lipid peroxidation in liver tissue, and enhanced alcohol oxidation. GHT treatment up-regulated IL-10 in the liver, whereas it down-regulated TNF- α , TGF- β , and Fas ligand.

Conclusion : From these results, GHT is presumed to work in the liver in protective roles not through the pathway of alcohol metabolism but mainly by anti-inflammation activity in our model.

Key Words: TNF- α , TGF- β , IL-10, alcohol, *Galhwahyejung-tang* (GHT)

Introduction

Alcoholic beverages have been used since the dawn of history. A great amount of alcohol have been consumed by adult, aged or even young around the world. According to the statistics of death rate, alcohol-related morbidity and mortality are serious problem both in Korea and United state¹⁾.

Excessive alcohol consumption can cause widespread damage to all tissue and organs of the body and potentially resulting in serious illnesses. Because the liver is the primary site of alcohol metabolism, damage

to the liver may be one of the most serious consequences of alcohol abuse²⁻⁴⁾.

As alcohol is broken down in the liver, a number of potentially dangerous by-products such as acetaldehyde and highly reactive molecules called free radicals are generated. Perhaps more so than alcohol itself, these products contribute to alcohol-induced liver damage. Most of the liver damage caused by alcohol is attributed to alcohol metabolism and the by-products of that metabolism and also to the inflammation that is induced secondarily by these same compounds⁵⁻⁷⁾.

Use of herbal drugs in the treatment of liver diseases has a long history, especially in Eastern medicine⁸⁾. GHT is the representative prescription for alcoholic liver disease, which is first prescribed by Li Donghen (李東垣) of Jin (金) and Yuan (元) Dynasty, has effects of perspiration(發汗), regulating urine (利小便) and is

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used for indulgence in drinking (飲酒過傷), vomiting the phlegm (嘔吐痰逆), tremor of the limbs (手足戰搖), mental confusion (精神昏亂), anorexia (飲食減少)⁹⁾.

In this study, we aimed at how GHT gives the benefits related with protective effects on alcohol-induced damage in liver.

Materials and Methods

1. Materials

Medicinal herb were purchased from Daejeon Oriental Medical Hospital. The composition of GHT, 1 day dosage (130 g) of the formula for a human adult was mixed with 1 L of distilled water and left for 1 h at room temperature, and the whole mixture was then boiled for 2 h. The GHT extract was then filtered and freeze dried. The yield of GHT extract was 9.5% (w/w) in terms of the dried medicinal herbs. The composition of GHT formula was mentioned in the table 1.

DNA taq polymerase was obtained from Bioneer (Cheongwon, Korea). M-MLV reverse transcriptase was obtained from promega (Madison, USA). TRIzol[®] reagent was obtained from Gibco (Maryland, USA). Rest of the reagents were purchased from Sigma (ST. Louis, U.S.A.).

2. Experimental animal treatments

Six-week old female Sprague-Dawley rats were purchased from commercial animal breeder (Daehan BioLink, Korea). After one week of acclimation, the rats were used for this experiment. The rats were housed in an environmentally controlled room at $22 \pm 2^\circ\text{C}$, relative humidity at $55 \pm 10\%$ and 12 h light/dark and fed with commercial pellets (Samyang Feed, Korea) and tap water *ad libitum*.

1. Acute model

Twenty four Sprague-Dawley rats were divided into 4 groups of 6 animals each. The rats in treatment group were administrated with GHT at 12 h and 4 h before alcohol administration. Then, the rats in group 2 were administrated orally with 40% ethanol and rats in group 3 and group 4 were administrated orally with 40% ethanol containing GHT (200 mg/kg and 600 mg/kg) at a dose of 10 ml/kg, whereas the rats in group 1 were given a single dose of distilled water. After 2 h, rats were anesthetized and blood was collected.

2. Chronic model

Twenty four Sprague-Dawley rats were divided into 4 groups of 6 animals each. Rats in group 2 were

Table 1. Prescription of *Galhwahyejung-tang* (GHT)

General Name	Part used	Relative Amount (g)
<i>Pueraria thunbergiana</i>	Flos	10
<i>Amomum villosum</i>	Fructus	10
<i>Amomum kravanh</i>	Fructus	10
<i>Citrus unshiu</i>	green Pericarpium	6
<i>Atractylodes macrocephala</i> Rhizoma	Rhizoma	4
<i>Zingiber officinale</i>	Rhizoma	4
<i>Massa medicata</i>	fermentata	4
<i>Alisma plantago-aquatica var orientale</i>	Rhizoma	4
<i>Panax ginseng</i>	Radix	3
<i>Polyporus umbrellatus</i>		3
<i>Poria cocos</i>		3
<i>Citrus unshiu</i>	Pericarpium	3
<i>Aucklandia lappa</i>	Radix	1
Total amount		65

administrated orally with 40% ethanol and rats in group 3 and group 4 were administrated orally with 40% ethanol containing GHT (200 mg/ml and 600 mg/ml) at a dose of 10 ml/kg, whereas the rats in group 1 were given a dose of distilled water once a day for 2 weeks. On day 7, after 2 h ethanol administration, the rats were anesthetized and bled. On day 14, after 2 h ethanol administration, the rats were anesthetized with ether and blood was collected. Their liver were removed and frozen rapidly for mRNA analysis.

3. Determination of serum alcohol level

For the analysis of serum alcohol level, blood was centrifuged for 15 min at $1500 \times g$. The serum was removed and preserved in deep freezer until assayed. Reaction buffer, 20 mM glycine buffer(pH 9.2) containing 72 mM semicarbazide was mixed 0.5 mM NAD⁺ and 36 unit/ml yeast alcohol dehydrogenase, and added 10 ul serum. Absorbance determined at 340 nm after standing for 10min at room temperature.

4. Serum biochemical analysis

The levels of serum aspartate transaminase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), and glucose were determined by using Auto Chemistry Analyzer (Olympus, Japan).

5. Determination of lipid peroxides

Rat liver were excised soon after the rats were killed and washed in saline. The liver homogenate were prepared at the ratio of 0.2 g organ to 1.8 ml of 1.15%(W/V) KCl. Thereafter, 0.13 ml homogenate was mixed with 0.08 ml of 1% H₃PO₄ and 0.26 ml of 0.67% TBA. The contents of each tube were heated in dry oven (100°C) for 45 min. After cooling in ice, 1.03 ml n-butanol was added to each tube and the contents were centrifuged for 10 min at $3000 \times g$. Absorbances of the supernatant were determined at 535 nm and 520 nm.

The concentration of lipid peroxides is calculated as A535-A520.

6. Gene expression of cytokine and enzyme

Total cellular RNA was isolated by the TRIzol[®] reagent (Gibco, Maryland USA) according to the manufacturer's instructions. The mRNA levels was quantified at 260 nm by spectrophotometer (Cary50, Varian, USA).

Total RNA was extracted from homogenized liver sample of SD female rats. The RNA (1 μ g) was reverse-transcribed (RT) into first strand cDNA in a RT mixture containing 2 μ g of 10 mM dNTPs mix, 1 μ g of oligo-dT primer (20 pmol/ μ l), 2 μ l of 100 mM DTT, 4 μ l of 5 \times RT buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 15 mM MgCl₂, RNase inhibitor 20 U), 1 μ l of M-MLV RT (200 U/ μ l; Promega, U.S.A) and 2 ul of DDW. The RT mixture was incubated at 42°C for 60 min, heated to 95°C for 5 min to inactivate the reverse transcriptase, and chilled to 4°C for 5 min. A portion of the RT product (1 μ l) was then subjected to the polymerase chain reaction (PCR) in a DNA thermal cycler (TaKaRa, Tokyo, Japan).

To determine the expression pattern of ADH, ALDH, CYP2E1, TNF- α , Fas ligand, TGF- β , IL-10 and iNOS mRNA in rat liver, 1 μ l of cDNA was amplified by a thermal cycler (TaKaRa, Japan) using the primers (Table 2). The PCR mixture was made as followings; 1.5 units of Taq DNA polymerase (Bioneer, Korea), 3 μ l of 10 mM dNTPs, 3 μ l of 10 \times PCR buffer, 1 μ l of 10 pmol sense and antisense primers, and 3 μ l of cDNA in 19.7 μ l of ultra distilled water.

PCR amplification was carried out in the thermal cycler using a protocol of initial denaturing step at 95°C for 10 min; then 35 cycles at 95°C for 1 min (denaturing), at 60°C for 40 s (annealing), and at 72°C for 10 min. The PCR products were run on a 1% agarose gel in 0.5 \times TBE buffer.

Table 1. Oligonucleotide Sequences of Primers

Gene	Primer	Sequence	Product size (bp)
β -actin	Sense Antisense	gtggggcgccccaggcacca ctcctaattgtcacgcacgattc	539
ADH	Sense Antisense	gatttccctggactgtgc ggcatgggtcaccagtaagt	255
ALDH	Sense Antisense	cctgcaactgaggaggtcat ccacacacccaatagggtc	248
CYP2E1	Sense Antisense	catggctacaaggctgtcaa tggcctttggtcttttgag	258
iNOS	Sense Antisense	fggtggtgacaagcacattt ctgagttcgtccccttctcc	229
TNF- α	Sense Antisense	ctcccagttctcttcaagg tggaaagactctcccagta	195
IL-10	Sense Antisense	tcttggaaaacctggttg tcttctccaagaccatga	376
TGF- β	Sense Antisense	tgagtggctgtctttgacg ttctctgtggagctgaagca	310
Fas ligand	Sense Antisense	actccgtgagttcaccaacc caagactgaccccgaagta	267

7. Statistic analysis

Results were expressed as the mean \pm standard deviation (SD). Statistical analysis of the data was carried out by Student's t-test. A difference from the respective control data at the levels of $p < 0.05$ was regarded as statistically significant.

Results

1. Rate of alcohol oxidation

All rat's blood were extracted on 2 h, day 7, and day 14 while administration of alcohol with once every day. On 2 h and day 7, serum alcohol level did not show any change between control and two GHT treatments, while it was slightly lower in GHT treatment groups than control group. The results were described in Fig. 1.

2. Serum biochemical analysis

After alcohol administration, all animal's peripheral

bloods were sampled via orbital vein or abdominal aorta. Serum AST level in GHT groups were 118 U/L and 117 U/L whereas serum AST level in control groups was 160 U/L. LDH level also significantly decreased by normal level by GHT administration. Between control and GHT treatment, there was no significant difference on ALT and glucose level. The results were described in Fig. 2, 3, 4.

3. Analysis of lipid peroxides

To determine the effect of GHT on lipid peroxidation induced by alcohol, Sprague-Dawley rats were killed on the 14th day. The lipid peroxidation in GHT groups was decreased compared with control group. The results were described in Fig. 5.

4. Gene expression of enzyme and cytokine

To examine the protective effect of GHT on alcohol-induced liver damage, we measured mRNA expression

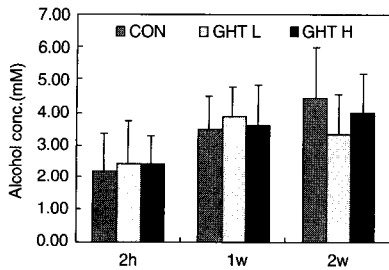


Fig. 1. Serum alcohol level in rats administrated 40% ethanol. CON, 40% ethanol only; GHT L, 40% ethanol containing 200 mg/kg of GHT; GHT H, 40% ethanol containing 600 mg/kg. The values were expressed as the mean \pm SD.

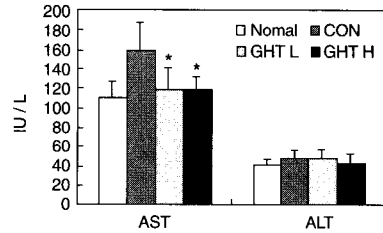


Fig. 2. Serum AST and ALT level in rat administrated with 40% ethanol for 2 weeks. CON, 40% ethanol only; GHT L, 40% ethanol containing 200 mg/kg of GHT; GHT H, 40% ethanol containing 600 mg/kg of GHT. The values were expressed as the mean \pm SD. *: $p < 0.05$, significant differences compared with the group administrated 40% ethanol only.

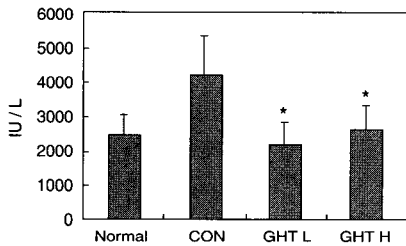


Fig. 3. Serum LDH level in rat administrated with 40% ethanol for 2 weeks. CON, 40% ethanol only; GHT L, 40% ethanol containing 200 mg/kg of GHT; GHT H, 40% ethanol containing 600 mg/kg of GHT. The values were expressed as the mean \pm SD. *: $p < 0.05$, significant differences compared with the group administrated 40% ethanol only.

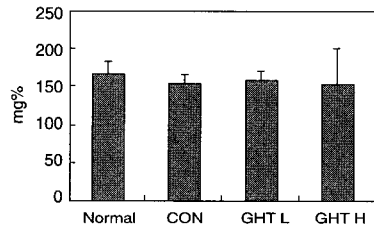


Fig. 4. Serum glucose level in rat administrated with 40% ethanol for 2 weeks. CON, 40% ethanol only; GHT L, 40% ethanol containing 200 mg/kg of GHT; GHT H, 40% ethanol containing 600 mg/kg of GHT. The values were expressed as the mean \pm SD.

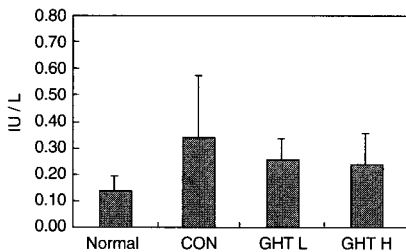


Fig. 5. Effects of GHT on lipid peroxides in rat administrated with 40% ethanol for 2 weeks. CON, 40% ethanol only; GHT L, 40% ethanol containing 200 mg/kg of GHT; GHT H, 40% ethanol containing 600 mg/kg of GHT. The values were expressed as the mean \pm SD.

of enzyme and cytokine from liver tissue of Rats. Expression of ADH, ALDH, CYP2E1, TNF- α , Fas ligand, TGF- β , IL-10 and iNOS genes were amplified and qualitatively analyzed by RT-PCR.

1) Enzyme expression related with alcohol metabolism

ADH and ALDH were up-regulated by alcohol administration, but difference between control and GHT treatment groups was not shown. No difference was shown in CYP2E1 gene expression compared with control group. The results were described in Fig. 6.

2) Cytokine expression related with inflammatory reaction

In GHT treated groups, TNF- α gene expression were slightly decreased compared with control group whereas IL-10 gene expression were up-regulated significantly. TGF- β and Fas ligand gene expressions in GHT groups were decreased markedly compared with control group. The results were described in Fig. 7.

Discussion

When alcohol is consumed, it passes from the stomach and intestines into the blood, a process referred to as absorption. Most of the alcohol is metabolized in the liver. Ethanol is metabolized by three enzyme

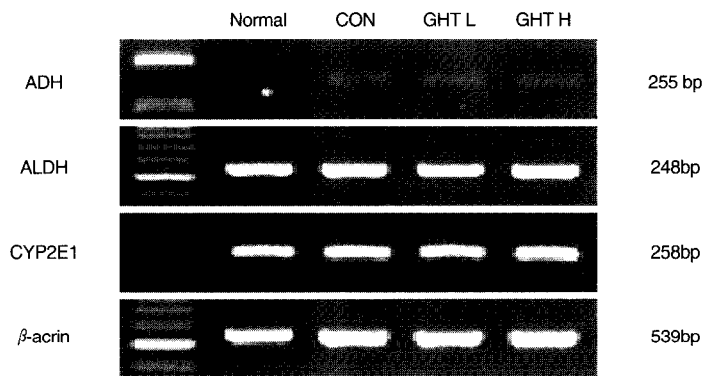


Fig. 6. Expression of mRNA related with alcohol metabolism was measured by RT-PCR in rat administrated with 40% ethanol for 2 weeks. CON, 40% ethanol only; GHT L, 40% ethanol containing 200 mg/kg of GHT; GHT H, 40% ethanol containing 600 mg /kg of GHT.

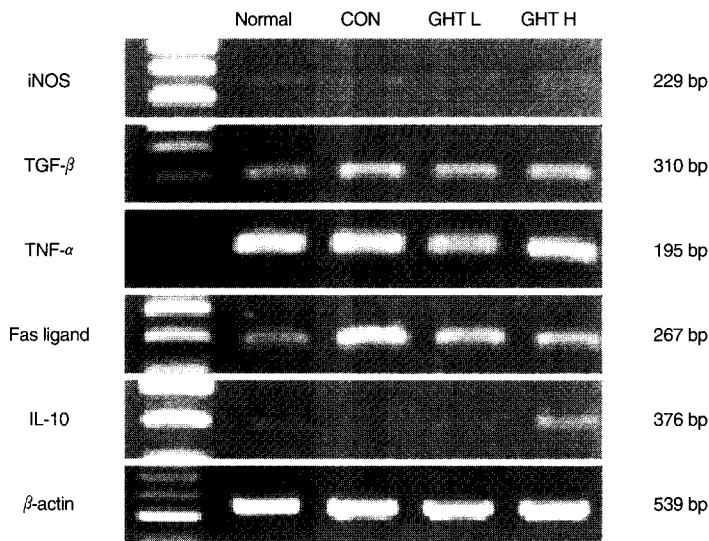


Fig. 7. Expression of mRNA related with inflammatory reaction was measured by RT-PCR in rat administrated with 40% ethanol for 2 weeks. CON, 40% ethanol only; GHT L, 40% ethanol containing 200 mg/kg of GHT; GHT H, 40% ethanol containing 600 mg/kg of GHT.

pathway including alcohol dehydrogenase in cytosol, microsomal enzyme oxidizing system (MEOS) and peroxisomal oxidation. All three systems produce acetaldehyde which is highly toxic to the body, even in low concentration. Normally, however, the enzyme ALDH rapidly oxidizes acetaldehyde to acetate. Most of the acetate travels through the bloodstream to other parts of the body, where it can enter other metabolic cycles that produce energy or useful molecules¹⁰.

The MEOS oxidizes alcohol to acetaldehyde by means of a cytochrome called CYP2E1, which is found in the endoplasmic reticulum of liver cells. Normally functioning at a low level, CYP2E1 is stimulated to a higher level by the presence of alcohol. Thus, the MEOS becomes increasingly important as alcohol consumption becomes heavier and more chronic¹¹.

In addition to these metabolic effects, the oxidation of ethanol results in the formation of a variety of reactive molecules, called free radicals. Much of the direct cell damage that occurs during alcoholic liver disease is believed to be caused by free radicals. Free radicals are highly reactive molecular fragments that frequently contain oxygen. Small quantities of free radicals are produced as normal by-products of various metabolic processes. These fragments are quickly scavenged by nature protective molecules in the cell, called antioxidants. However, when free radicals are produced in excess or when antioxidant defenses are impaired, the free radicals may interact destructively with vital cell constituents, potentially causing death of the cell¹².

In this study, GHT treatment did not decrease serum alcohol level in rats for short-term (2 h, 1 week). However the concentrations of serum alcohol were slightly lowered in GHT treatment group compared with control group for long-term (2 weeks). In enzyme expression related alcohol metabolism by RT-PCR, ADH and ALDH were up-regulated by alcohol administration, but difference between control and GHT

treatment groups was not shown. GHT did not affect on enzyme gene expression related alcohol metabolism. so we presume that GHT don't affect directly alcohol oxidation.

Oxidative damage is an important feature of human alcoholic liver disease¹³. Protein modifications induced by oxidative damage and particularly the formation of protein adduct with lipid peroxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (HNE) can be detected in the liver of ethanol-fed rat¹⁴. Aldehyde-modified proteins are highly immunogenic and both monoclonal and polyclonal antibodies towards epitopes derived from different lipid peroxidation products have been characterized in recent years^{15,16}. GHT reduced lipid peroxidation induced by ethanol. So we presume that GHT possesses antioxidant potency.

AST presents two isozymes, one located in the cytoplasm and the other in the mitochondria¹⁷. The presence of these enzymes outside the cell represents damage to the hepatic cell. Serum AST and LDH activity decreased significantly in GHT treatment groups. These results indicate that GHT have liver protective effect on ethanol induced hepatotoxicity. In vitro data support this conclusion.

IL-10 is known as an anti-inflammatory cytokine, level of IL-10 mRNA is lower in the group exhibiting inflammatory liver injury¹⁸. The kupffer cell is the predominant immune-effector cell in the liver that, in response to inflammatory mediator, generates TNF- α and other pro-inflammatory cytokines¹⁹. A combined role for TNF- α and Cox-2 in alcoholic liver injury in the intragastric feeding rat model has been previously shown²⁰. Fas ligand expression is up-regulated by acetaldehyde and up-regulation of Fas ligand expression is one of ethanol induced apoptosis mechanisms²¹. GHT treatment up-regulated IL-10, whereas down-regulated TNF- α and TGF- β . GHT also down-regulated Fas

ligand. This results indicate that GHT decreases liver inflammation induced by alcohol and protects apoptosis of hepatocyte via Fas ligand pathway.

From all these results, GHT has protective potency on alcoholic liver injury through inhibition of inflammation and apoptosis induced by alcohol-metabolic products. This study strongly support the clinical validity of GHT which we have prescribed for patients suffering from alcoholic liver disease.

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