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Original Article

Effect of *Galhwahyejung-tang* (GHT) on Alcohol-induced Oxidative Stress in Rats

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Objectives: This study was aimed to investigate the effects of *Galhwahyejung-tang* (GHT) on alcohol-induced oxidative stress in rat model.
Methods: Twenty SD rats were orally administrated with 40% ethanol (mL/kg) combined with GHT (50, 100, 200mg/kg) or distilled water for 2 weeks. Biochemistry in blood, malondialdehyde (MDA), total reactive oxygen species (ROS), and total antioxidant capacity (TAC) in serum, liver, brain, and kidney were determined.
Results: GHT treatment significantly ameliorated the alcohol-induced alteration of hepatic enzyme; especially AST and ALT. GHT treatment also ameliorated the increase of MDA in liver, ROS level in serum and brain. GHT treatment reduced the depletion of antioxidant capacity in serum and brain.
Conclusion: These results that GHT has antioxidant properties explaining the relevance of clinical application and its partial mechanisms of GHT.

Key Words : Alcohol, oxidative stress, *Galhwahyejung-tang*, traditional Korean medicine

Introduction

Alcohol is a very popular beverage worldwide. However, the physical/psychological diseases and social problems related to alcohol abuse are very common[1)]. According to the report for Korea National Statistical Office, the number of alcohol-associated deaths in 2007 was 4,701, and alcohol is the second and third cause of liver cirrhosis and hematoma, respectively[2)]. Therefore, controlling alcohol abuse or treating alcohol-induced disorders have become important social and medical issues[3)].

Alcohol absorbed is generally metabolized mainly in the liver using various enzymes, and then is eliminated before induction of toxicity[4)]. However, over-consumption of alcohol induces many disorders including intoxication, dehydration, and ultimately alcohol poisoning in short term cases, while it leads to changes of metabolism in the liver and brain, and possible addiction in the long term case[5)]. The main mechanism of alcoholic toxicity is supposed to be oxidative stress[6)].

On the other hand, there have been many traditional formulae used for alcoholic disorders in traditional Korean medicine[7,8)]. *Galhwahyejung-tang* (GHT) is a typical herbal formula used to treat alcohol-induced symptoms such as vomitting, tremor of the limbs, anorexia, and mental confusion[9)]. So far, several

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studies have presented the effects of GHT, especially on protection of liver from fatty liver and hepatitis using animal models[10,11]. The main focus of these studies was on liverfunction; so far no study has been done to test the effects of GHT on other organs such as brain under alcohol toxicity conditions.

The present study aimed to investigate the anti-oxidant effect of GHT in serum and also in other tissues like liver and brain.

Materials and Methods

1. Materials and Preparation of GHT

Absolute ethanol was purchased KGaA (Darmstadt, Germany). 2,2-diphenyl-1-picrylhydrazylradical (DPPH), N,N-diethyl-para-phenylendiamine (DEPPD) sulfate and ferrous sulfate, myoglobin, Trolox, and ABTS were purchased from Sigma Chemicals (St. Louis, MO, USA). Hydrogen peroxide was obtained from Junsei (Tokyo, Japan). All other chemicals and experiment instrument commercially available.

Medical herbs for GHT formula (Table 1) were purchased from Jeong-Seoung oriental herb company (Daejeon, Korea). To extract GHT, 130g of herb mixture were boiled for 2 h with 1.3 L of distilled water. After filtering, the GHT decoction was freeze-dried using a rotary evaporator. GHT dried extract thus obtained was 14.25 g (final yield 9.5%).

2. Animals and experimental design

Specific pathogen free 6-week-old Sprague Dawley (SD) rats were purchased from a commercial animal breeder (Orient Bio, Gyeongido, South Korea). Rats were acclimatized for 1 week and housed in an environment-controlled room at 22±2℃ with a 12 h light/dark cycle, and provided commercial pellets (Orient Bio) and tap water ad libitum. Twenty-five SD rats were randomly divided into five groups of fiveanimals. 40% ethanol (mL/kg) was treated orally for 2 weeks, except for the normal group. GHT (50, 100, 200 mg/kg) or distilled water was administered two hours before ethanol treatment.

The animals were sacrificed on the fifteenth day, and the liver, kidney and spleen were isolated and weighed. Liver, kidney and brain tissues were stored for estimation of malondialdehyde (MDA), total reactive oxygen species (ROS), and total antioxidant capacity (TAC).

Animal experiments were conducted in accordance with the Guide for Care and Use of Laboratory

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

General Name	Using part	Amount (g)	Composition rate (%)
Pueraia thunbergiana	Flos	10	15
Amomum villosum	Fructus	10	15
Citrus unshiu	Green Pericarpium	6	9
Atractylodes macroephala	Rhizoma	4	6
Zingiber officinale	Rhizoma	4	6
Massa medicata	Fermentata	4	6
Alisma olantago-aquatica var orientale	Rhizoma	4	6
Panax ginseng	Raix	3	5
Poria cocos		3	5
Citrus unshiu	Pericarpium	3	5
Aucklandia lappa	Raix	1	2
Total amount		65	100

Animals published by the U.S. National Institutes of Health[12].

3. Serum biochemical analysis

Blood was collected via the abdominal aorta under ether anaesthesia onfinal day of the experiment. Serum was collected following 60 minutes of blood clotting. Serum levels of alanine transaminase (ALT), aspartate (AST), and alkaline phosphatase (ALP) were determined using an Auto Chemistry Analyzer (Chiron, Emeryville, CA, USA).

4. Determination of Malondialdehyde (MDA)

Lipid peroxidation levels in the liver, kidney and brain tissue were determined using the method of thiobarbituric acid reactive substances (TBARS)[13]. The concentration of TBARS was expressed as uMMDA/g tissue using 1.1.3.3-tetraethoxypropane (TEP) as a standard. Briefly, 0.2 g liver tissue was homogenized in 2 mL ice-cold 1.15% KCl, and 0.13 ml homogenate was mixed with 0.08 ml 1% phosphoric acid and 0.26 ml 0.67% thiobarbituric acid (TBA). After heating the mixture for 45 min in a dry oven (100℃, 1.03 ml n-butanol was added followed by a vigorous vortexing and centrifugation at 3,000 rpm for 15 min. The absorbance of the upper organic layer was measured at 535 and 525 nm with a spectrophotometer and compared with a TEP standard curve.

5. Determination of total reactive oxygen species (ROS)

Total ROS level in serum, liver and brain tissue were determined according to the method of Hayashi[14]. Briefly, hydrogen peroxide was used for generating a calibration curve as standard. DEPPD solution and ferrous sulfate solution (100 ug/mL of DEPPD and 4.37 uM of ferrous sulfate was dissolved in the 0.1 M sodium acetate buffer separately) were prepared beforehand. Five uL of standard solution or 1:10 diluted liver or brain homogenate supernatant were added to 140 uL of 0.1 M sodium acetate buffer (pH 4.8) in each well of 96-well plates. After 5 minutes incubation at 37℃ 100 uL of DEPPD and ferrous mixture solution (scale factor of 1/25 was used) were added to each well. Similarly, 0.1 g organ tissues were homogenized in 1 mL ice-cold RIPA buffer and the same method was followed as described above. The level of ROS was determined at 505 mm using a spectrophotometer with catalytic capability for transition metals, and calculated as equivalent to levels of hydrogen peroxide (1 Unit = 1 mg H_2O_2/L).

6. Determination of total antioxidant capacity (TAC)

TAC levels were determined according to the method of Kambayashi[15]. 90 uL of 10 mM phosphate-buffered saline (pH 7.2), 50 uL of myoglobin solution (45 uM), 20 uL of 3 mM ABTS solution, 20 uL of diluted serum sample or tissue sample (liver and brain) and Trolox was added to 96-well microplate and well mixed at 25℃ Then 20 uL of H_2O_2 was added to each well, and incubated for 5 min. The absorbance was read using a plate reader at 600 nm (Molecular Device Corp., USA).level of TAC was expressed as Trolox equivalent antioxidant capacity.

7. Statistical analysis

Results were expressed as the mean ± 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. Effect on body and organ weight

40% ethanol treatment slightly decreased body weight and liver weight, and significantly spleen weight

($p < 0.01$) of rats. Co-treatment with GHT slightly ameliorated these changes, but didn't reach statistical significance. The absolute and relative weight of body and organ weight was summarized (Table 2).

2. Effect on biochemical parameters

40% ethanol treatment significantlyelevated ALT and moderately AST and ALP. In contrast, GHT ameliorated elevations in ALT ($P < 0.01$) and AST ($P < 0.01$), especially in the GHT 200 group (Fig 1).

3. Effect on MDA levels

40% ethanol treatment significantly elevated MDA levels in the liver, but not in the kidney or brain. Significant change in MDA ($p < 0.05$) was seen in GHT group. No significant change was observed in kidney and brain tissue on GHT (Fig 2).

4. Effect on total ROS level

40% ethanol treatment significantly elevated ROS level in serum, liver, and brain. GHT slightly ameliorated these elevations in all tissues. The GHT 200 group showed significantly lower ROS level in serum ($p < 0.05$) and brain ($p < 0.01$). No significant difference was observed in the liver (Fig. 3).

5. Effect on TAC level

40% ethanol treatment moderately depleted antioxidant capacity in serum and the brain. The depletion of TAC in serum was significantly ameliorated by 50 and 100 mg of GHT ($p < 0.01$) and 200 mg of GHT treatment ($p < 0.05$). In brain tissue, 200 mg of GHT treatment significantly inhibited depletion of TAC ($p < 0.05$). However, no difference was observed in the liver tissues of the control and GHT groups (Fig. 4).

Discussion

Oxidative stress is defined as a combination of excessive generation of reactive oxygen species (ROS) and decreased antioxidant defenses[16]. Over consumption of alcoholicbeverages causes production of ROS such as superoxide, hydrogen peroxide, or hydroxyl radicals as well as distortion of various protective systems including free radical scavengers, superoxide dismutase (SOD), catalase, and the glutathione oxidation/reduction system[17]. This unbalanced state is a major pathogenic mechanism contributing to alcoholic symptoms and damage to various organs.

GHT is a typical herbal formula that has been prescribed for patients with various symptoms associated with alcohol abuse. Accordingly, it is proposed that GHT has antioxidant pharmaceuticalproperties. One study previously revealed its antioxidant effects especially against ethanol-administrated rat stomach, including inhibition of lipid peroxidation, and maintaining of glutathione and SOD contents[18]. We herein adapted a two-week ethanol treatment in a rat

Table 2. Body and organ weights

	Absolute weight (g)			Relative weight (%)		
	Body	Liver	Spleen	Body	Liver	Spleen
Naive	352.9±30.5	12.40±1.89	0.88±0.09	-	3.50±0.29	0.25±0.04
40% ethanol	323.3±7.1	10.31±0.39	0.67±0.09##	-	3.18±0.07	0.19±0.03#
GHT 50	321.1±7.9	9.88±0.95	0.55±0.07	-	3.08±0.30	0.17±0.02
GHT 100	320.3±11.1	9.61±0.61	0.67±0.09	-	3.00±0.13*	0.20±0.03
GHT 200	316.9±3.3	10.00±0.67	0.71±0.12	-	3.16±0.22	0.22±0.04

The rats were administered with 40% ethanol, combined with GHT (50, 100, 200 mg/kg) or distilled water for 14 days. On the final day of the experiment, body and organ weight were measured. Relative organ weight was calculated against body weight. Data are expressed as mean ± SD (n = 5). #P < 0.05, ##P < 0.01 compared to the naive group.

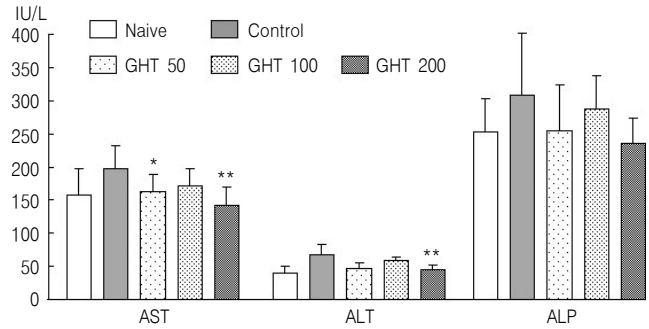

Fig. 1. Changes in serum biochemical parameters.

Rats were administrated 40% ethanol with/without GHT for two weeks. On the last day, AST, ALT, and ALP value in serum were measured. Data are expressed as mean ± SD (n = 5). $*P < 0.05$, $**P < 0.01$ compared to the control group.

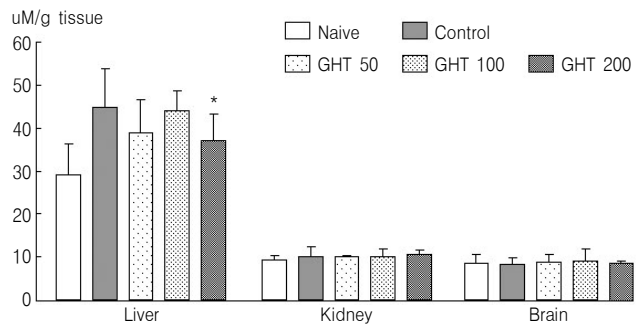

Fig. 2. Change of MDA level.

Rats were administrated 40% ethanol with/without GHT for two weeks. MDA values in liver, kidney, and brain serum were measured. Data are expressed as mean ± SD (n = 5). $*P < 0.05$ compared to the control group.

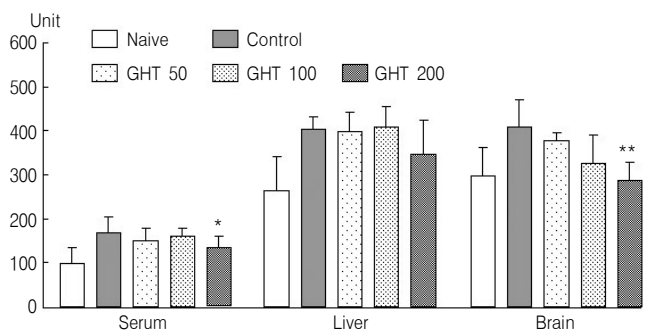

Fig. 3. Change of ROS level.

Rats were administrated 40% ethanol with/without GHT for two weeks. ROS value in serum, liver, and brain were measured. Data are expressed as mean ± SD (n = 5). $*P < 0.05$, $**P < 0.01$ compared to the control group.

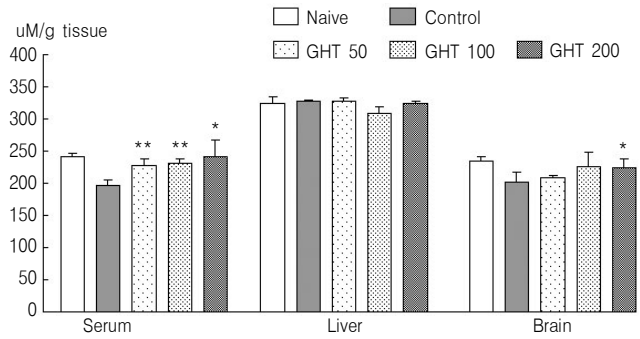

Fig. 4. Change of TAC level.

Rats were administrated 40% ethanol with/without GHT for two weeks. TAC value in serum, liver, and brain were measured. Data are expressed as mean ± SD (n = 5). *P < 0.05, **P < 0.01 compared to the control.

model, and then investigated the antioxidant effects of GHT in several organ tissues.

40% ethanol treatment for 2 weeks induced mild hepaticcellular injury resulting in elevation of AST and ALT. It also elevated the levels of MDA (product of lipid peroxidation) and ROS in the liver, as already reported. Antioxidant enzymes quickly eliminate the free radicals generated during the process of ethanol metabolism. However, when the generated ROS exceeds the ability of the cell to remove them, there occurs lipid peroxidation and depletion of antioxidant enzymes leading to hepatocyte damage. In the current study, co-treatment with GHT protected liver tissues from cellular damage and alterations of MDA level, especially in the high dose group. This result is in accordance with other data from animal and clinical examinations[11,19].

Although we didn't examine all antioxidant enzymes, two-week ethanol administration didn't induce depletion of TAC in the liver. TAC was radically lowed in serum and brain tissue. In accordance with that, ROS level became very high in serum and the brain. GHT treatment significantly improved these abnormal alterations of TAC and ROS levels, in particularlyserum and brain tissue. These results support that GHT may show more antioxidant actions for serum and brain compared to the liver.

The liver is the central organ to metabolize alcohol. So, it has been thought that the liver is most easily injured by alcohol consumption[20]. The liver usually maintains sufficient antioxidant enzymes, so short term alcohol abuse could be tolerable to the liver. In contrast, other organs including the stomach, blood, or brain might be more susceptible to alcohol-induced oxidative stress.

In fact, the main clinical applications of GHT are not to treat liver-specificsymptoms such as jaundice or ascites, but to treat acute hangover symptoms like vomitting, anorexia, tremor of the limbs, and mental confusion[9,21].

Taken together, this study showed experimental evidence for antioxidant propertiesof GHT. Additionally, it is strongly proposed that the clinical relevance of GHT is associated with its antioxidant action in various organs, including serum and brain predominantly.

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