Favorable Hepatoprotective Effects of Gongjin-dan on the Acute Ethanol-induced Liver Damaged C57BL/6 Mice

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To observe the potential hepatoprotective effects of Gongjin-dan on the acute ethanol (EtOH)-induced liver damages in C57BL/6 mice with its possible action mechanisms. EtOH-mediated acute hepatic damages were induced by oral administration of EtOH total 3 doses. The changes on the body weight, liver weight, albumin, TG, AST, ALP, ALT, hepatic TG contents, hepatic antioxidant defense system, TNF-α, CYP 2E1 activity and mRNA expressions of hepatic lipogenic genes - SREBP-1c, SCD1, ACC1, FAS, PPARγ and DGAT2 or genes involved in fatty acid oxidation - PPARα, ACO and CPT1 were observed with final liver histopathological inspections after 15 days of continuous administration of silymarin 200 mg/kg, Gongjin-dan (GJD) 400, 200 and 100 mg/kg. The results were compared with silymarin 200 mg/kg treated mice. Marked decreases of body and liver weights, increases of serum AST, ALT, Albumin and TG levels, hepatic TG contents, TNF-α level, CYP 2E1 activity and mRNA expressions of hepatic lipogenic genes or decreases mRNA expressions of genes involved in fatty acid oxidation were observed with histopathological changes related hepatosteatosis increases of immunolabelled hepatocytes, as the results of a binge drinking of EtOH in the present study. Also destroys of hepatic antioxidant defense systems were demonstrated in EtOH control mice as compared with intact vehicle control mice, respectively. The results suggest that oral administration of 400, 200 and 100 mg/kg of GJD favorably protected the liver damages from acute mouse EtOH intoxications.

keywords : Gongjin-dan, Acute ethanol-induced liver damaged, hepatoprotective

Introduction

The liver has been frequently targeted by a number of toxicants because it is actively involved in metabolic functions[1)].

Ethanol (EtOH) administration causes accumulation of reactive oxygen species (ROS), including superoxide, hydroxyl radical, and hydrogen peroxide[2)]. The ROS/free radicals is an intracellular metabolic process and these species are known to cause oxidative damages to the membrane lipids, proteins, and nucleic acids[3)]. Reduced glutathione (GSH) is the dominant defense against ROS/free radicals in different tissues of the body[4)]. Many chemicals and drugs can increase ROS/free radicals formation in specific body organs. EtOH has been revealed by investigators as an individual source for ROS production[5)].

Silymarin is one of powerful antioxidants to protect liver cells (and other cells in the body and brain) from toxins. On the EtOH-induced liver damages, anti-oxidant effects of silymarin have been well-documented and used as control reference drugs on EtOH-induced liver injuries[6-8)].

Gongjin-dan (GJD), a traditional Korean polyherbal prescription is one of the most famous tonic agents. Although possible hepatoprotective effects of Gongjin-dan on alcoholic liver damages in rats have been already reported by other investigators, more detail systemic hepatoprotective effects of Gongjin-dan seem to be needed to focus on the action mechanisms like anti-inflammatory, antioxidants, anti-steatosis properties, and particularly anti-tumor necrosis factor (TNF) production and decreasing lipid accumulation that have played important roles in the pathogenesis of alcoholic liver disease[5,6,9)].

In this study, we used Gongjin-dan obtained from Daegu Korean Hospitals, Daegu Haany University (Daegu, Korea) (GJD).

Materials and Methods

1. Animals and husbandry

Total seventy healthy males SPF/VAF CrljOri: C57BL/6 mice (OrientBio, Seungnam, Korea; 6-week old upon receipt;

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Body weight ranged in 18~20 g upon receipt) were used after acclimatization for 10 days. Animals were allocated four or five per polycarbonate cage in a 20-25℃ temperature and 50-55 % humidity controlled room. Light : dark cycle was per 12 hr, standard rodent chow (Samyang, Korea). Animal experiments approved by the Institutional Animal Care and Use Committee in Daegu Haany University (Gyeongsan, Gyeongbuk, Korea) [Approval No DHU2014-048, 2014. 07. 07]. Six groups, eight mice in each group(Table 1), total forty-eight mice were selected based on their body weight after 10 days of acclimatization(average 22.18±1.29 g, ranged in 19.5~23.8 g). EtOH-mediated acute hepatic damage was induced by oral administration of EtOH 5 g/kg, 12 hr-intervals, total 3 doses. Isocalorical maltose solution was orally administered in intact control mice instead of EtOH(Fig. 1).

Table 1. Experimental Design Used in This Study

Effects of GJD on the C57BL/6 mouse model of acute EtOH intoxication			
Group	Pretreatment	Group identification	Drug Treatment
Control	Isocalorical maltose	Intact control	Distilled water 10 ml/kg/day
Control	EtOH	EtOH control	Distilled water 10 ml/kg/day
Reference	EtOH	Silymarin	Silymarin 200 mg/kg/day
Active	EtOH	The highest dosages	GJD 400 mg/kg/day
Active	EtOH	The middle dosages	GJD 200 mg/kg/day
Active	EtOH	The lowest dosages	GJD 100 mg/kg/day

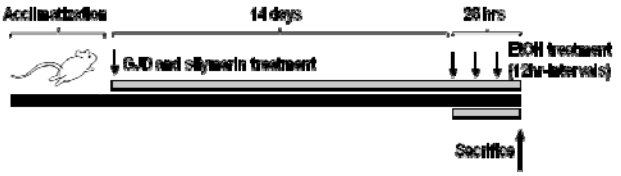

Fig. 1. Experimental Design Used in This Study.

2. Preparations and administration of test materials

GJD was obtained from Daegu Korean Hospitals, Daegu Haany University (Daegu, Korea) as listed follows, and used in this study. GJD was plastered using honey, and coated by gold plates(Table 2). It was stored in a refrigerator at -20 ℃ to protect from light and humidity. GJD was well suspended at least 40 mg/ml concentrations in distilled water and used of 400, 200 or 100 mg/kg based on the previous in vivo experiments[9]. Silymarin was obtained from Sigma-Aldrich (St. Louise, MO, USA) and used at a dose level of 200 mg/kg as reference control drug as listed follows[6]. Silymarin (200mg/kg) and three dosages of GJD (400, 200 and 100 mg/kg) were suspended in distilled water by homogenization for 3 min. It started at 14 days prior to first EtOH treatment orally administered once a day for 15 days, with a volume of 10 ml/kg. Instead of silymarin or GJD suspensions, equal volumes of distilled water were administered in Intact and EtOH control mice(Table 1).

Table 2. Composition of GJD Used in This Study

Herbs	Scientific Names	Amounts (g/pill)
Antler (Cervi Parvum Cornu)	Cervus elaphus Linne	0.683
Angelicae Gigantis Radix	Angelica gigas Nakai	0.683
Ginseng Radix	Panax ginseng CA Mey.	0.683
Corni Fructus	Cornus officinalis Sieb. Et Zucc	0.683
Rehmanniae Radix Preparata	Rehmannia glutinosa (Gaertner) Liboschitz	0.683
Moschus	Moschus moschiferus Linne	0.122
Mel	Apis indica Radoszkowski	2.506
Aurum Foil		0.006
Total	8 types	6.049

3. Induction of EtOH-mediated hepatic damage

Acute EtOH-induced toxicity of liver was induced by oral EtOH administration (0.8 g/ml concentration; Merck, Darmstadt, Germany) 5 g/kg, 12 hr-intervals, total 3 doses in a volume of 10 ml/kg, diluted as 6.74:3.25 (v/v) using distilled water[5,6]. First administration of EtOH was done at 30 min after 14 th material administration test. Last treatments of EtOH was conducted at 30 min after 15 th administration of GJD 400, 200 and 100 mg/kg, silymarin 200 mg/kg, respectively. Instead of EtOH, isocalorical maltose solution was orally administered in intact control mice.

4. Body and liver weight measurements

Body weight changes were measured at 1 day before initial test administration, a day of first test material administration, 1, 7 10, 14 days after initial silymarin or GJD administration using an automatic electronic balance (Precisa Instrument, Dietikon, Switzland). To diminish the individual differences, body weight during 15 days of experimental period were measured as (Body weight at the day of last test material administration - body weight at the day of first material administration). At sacrifice, liver weight was measured at g levels (absolute wet-weight). To reduce the differences from individual weight, relative weight (% of body weight) were measured too, using that equation such as [(Absolute wet-weight of liver / Body weight at sacrifice) × 100].

5. Measurement of serum biochemistry, hepatic TG contents and TNF-α levels

About 1 ml of venous blood was collected from the sacrifice's vena cava under anesthesia with 2~3 % isoflurane (Hana Pharm. Co., Hwasung, Korea) in the

mixture of 28.5 % O_2 and 70 % N_2O. All blood samples were centrifuged at 12,500 rpm for 10 min under cool temperature (4℃) using a clotting activated serum tube. After the serum albumin, asparatate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) levels were detected respectively using a blood biochemistrical auto-analyzer (Hemagen Analyst, Hemagen Diagnostic, Columbia, MA, USA). In addition, also serum triglyceride (TG) levels were measured using another type of automated blood biochemistrical analyzer (SP-4410, Spotochem, Kyoto, Japan) in this experiment.

The right lobe's liver tissue was homogenized in the equal volume of normal saline, extracted with a mixture of methanol and chloroform (1:2) as described previously[10]. To remove phospholipids, Zeolite (Sigma-Aldrich, St. Louise, MO, USA) was added. The resulting extract was dried by nitrogen and dissolved in Plasmanate (1 ml; Sigma-Aldrich, St. Louise, MO, USA). TG were measured using commercial kits (Kyowa Medex, Tokyo, Japan) enzymatically as previous methods[11] and expressed as mg/g tissue.

Liver samples were disintegrated in 5 volumes of radioimmuno- precipitation assay (RIPA) buffer. After 30 min incubation in ice, samples were centrifuged twice at 20,000×g for 15 min at 4 ℃. The resulting supernatants were used for assay. Contents of total protein were measured by previous method[12] using bovine serum albumin (Invitrogen, Carlsbad, CA, USA) as internal standard. The TNF-α levels were detected by enzyme-linked immunosorbent assay (ELISA) using a murine kit (BioSource International Inc.,Camarillo, CA, USA) and microplate reader (Tecan, Männedorf, Switzerland), and expressed as pg/mg protein.

6. Determination of CYP 2E1 Activity

AS a reaction catalyzed specifically by cytochrome P450 (CYP) 2E1, hydroxylation of p-nitrophenol to 4-nitrocatechol was determined colorimetrically[6]. Liver tissue was homogenized in 0.15 KCl and was spun at 10,000×g for 30 min. Microsomes were isolated by further centrifugation at 15,000×g for 60 min. Microsomal protein 300 ml was incubated for 5 min at 37 ℃, and absorbance at 535 nm was measured with 4-nitrocatechol as a standard using UV/Vis spectrometer (OPTIZEN POP, Mecasys, Daejeon, Korea) for the assay. The CYP 2E1-catalyzed p-nitrophenol hydroxylation was expressed as nM/min/mg protein.

7. Measurement of hepatic antioxidant defense systems

The separated liver tissues were weighed and homogenized in ice-cold 0.01 M Tris-HCl (pH 7.4), and centrifugated at 12,000×g for 15 min as described by Kavutcu et al.[13]. Liver lipid peroxidation concentrations were determined by estimating MDA using the thiobarbituric acid test at absorbance 525 nm, as nM of MDA/mg protein[14]. Contents of total protein were measured by previous method[12]. These homogenates were mixed with 0.1 ml of 25 % trichloroacetic acid (Merck, West Point, CA, USA), and centrifuged at 4,200 rpm for 40 min at 4 ℃. Glutathione (GSH) contents were measured at absorbance 412 nm using 2-nitrobenzoic acid (Sigma-Aldrich, St. Louise, MO, USA)[15]. H_2O_2 decomposition of in the catalase presence was followed at 240 nm[16]. Catalase activity was defined as the amount of enzyme required to decompose 1 nM of H_2O_2 per minute, at pH 7.8 and 25 ℃. The results were expressed as U/mg protein. The superoxide dismutase (SOD) activities' measurements were made according to Sun et al.[17]. The SOD estimation was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with nitrotetrazolium blue to form formazan dye. The SOD activity was then measured at 560 nm by degree of inhibition of this reaction, and expressed as U/mg protein. One unit of SOD activity is equal to the amount of enzyme which diminishes the initial absorbance of nitroblue tetrazolium by 50 % during 1 min.

8. Quantitiative RT-PCR

The RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA)5). Samples were treated with recombinant DNase I (DNA-free; Ambion, Austin, TX, USA) to remove contaminating DNA. The cDNA strand first was synthesized from total RNA and then mixture of the primers and cDNA products was amplified by PCR. The conditions of PCR amplification were 58 ℃ for 30 min, 94 ℃ for 2 min, 35 cycles of 94 ℃ for 15 sec, 60 ℃ for 30 sec, 68 ℃ for 1 min, and then 72 ℃ for 5 min. Finally, PCR products were separated on 0.8 % agarose gel. The analysis was carried out using gel imaging system (Bio-Rad, Hercules, CA, USA). Expression levels of sterol regulatory element-binding protein-1c (SREBP-1c), stearoyl-CoA desaturase 1 (SCD1), acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), peroxisome proliferator-activated receptor gamma (PPARγ), diacylglycerol acyltransferase 2 (DGAT2), peroxisome proliferator-activated receptor alpha (PPARα), acyl-CoA oxidase (ACO) and carnitine palmitoyltransferase 1 (CPT1) were calculated as percentage relative to Intact group using β-actin RNA as the internal control.

9. Histopathology

At 4 hrs after the last EtOH treatment, all of the mice were sacrificed. The samples from the left liver lateral lobes were fixed in 10 % neutral buffered formalin (NBF). They were also embedded in paraffin, sectioned (3~4 μm), stained with Hematoxylin and eosin (H&E) for general histopathology. After that the histopathological profiles of each sample were observed under light microscope (Nikkon, Tokyo, Japan). To more detail changes, numbers of hepatocytes which occupied by over 20 % of lipid droplets infiltrated in cytoplasm, were calculated using automated image analyzer (iSolution FL ver 9.1, IMT i-solution Inc., Vancouver, Quebec, Canada) as cells/1000 hepatocytes. At least 10 hepatocytes per each view field of liver, the percentage of fatty changed regions (%/mm^2 of hepatic parenchyma) and the mean diameters of hepatocytes (μm/hepatocytes) were calculated using automated image analyzer in both lateral and median lobes[18].

10. Immunohistochemistry

After the deparaffinization of the prepared hepatic histological paraffin sections, citrate buffer antigen (epitope) retrieval pretreatment were conducted[19]. Pre-heat the water bath with staining dishes containing 10 mM citrate buffers (pH 6.0) until the temperature reaches 95-100 ℃. Immerse the slides into the staining dish and place the lid loosely on top. Incubate for 20 min and turn off the water. Place the staining dish at room temperature and allow the slides to cool for 20 min. After the epitope retrievals, sections were immunostained using avidin-biotin complex (ABC) methods for nitrotyrosine (NT) and 4-hydroxynonenal (4-HNE)[19]. Endogenous peroxidase activity was blocked by incubate in 0.3 % H_2O_2 and methanol for 30 min. Non-specific binding of immunoglobulin was blocked with normal horse serum blocking solution (Vector Lab., Burlingame, CA, USA. Dilution 1:100) for 1 hr in humidity chamber. Primary antiserum was treated for overnight at 4 ℃ in humidity chamber, then incubated with biotinylated universal secondary antibody (Vector Lab., Dilution 1:50) and ABC reagents (Vectastain Elite ABC Kit, Vector Lab., Burlingame, CA, USA; Dilution 1:50) at room temperature for 1 hr in humidity chamber. Finally, reacted with peroxidae substrate kit (Vector Lab., Burlingame, CA, USA) for 3 min at room temperature. All sections were rinsed in 0.01 M PBS 3 times between each step. The cells showed stronger immuno-reactivities in the cytoplasm, over 20 %. Against each antiserum as compared with Intact control hepatocytes, the density was regarded as positive immunoreactive. The numbers of NT- and 4-HNE-positive cells were measured using a digital image analyzer among total 1000 hepatocytes, respectively[20].

11. Statistical analyses

All numerical data was expressed as mean±standard deviations (SD) of eight mice. Variance homogeneity was examined using the Levene test[21]. If Levene test indicated no significant deviations from variance homogeneity, the obtain data was analyzed by one way ANOVA test followed by least-significant differences (LSD) multi-comparison test to determine which pairs of group comparison were different significantly. In case of significant deviations from variance homogeneity were observed at Levene test, a non-parametric comparison test, Kruskal-Wallis H test was conducted. When significant difference is observed in the Kruskal-Wallis H test, the Mann-Whitney U (MW) test was conducted to determine specific pairs of group comparison, which are different significantly. Statistical analyses were conducted using SPSS for Windows (Release 14.0K, IBM SPSS Inc., Armonk, NY, USA)[22]. Test material treated rats were calculated to help the understanding the efficacy of test substances as follow [((Data of EtOH control-Data of Intact control mice)/Data of Intact control mice)×100] and [((Data of test material treated mice-Data of EtOH control mice)/Data of EtOH control mice) × 100], respectively.

Results

1. Changes in body weight

Significant ($p<0.01$) increases in body weight and body weight gains were observed in 15 th treatment day of test material in silymarin 200 mg/kg, GJD 100, 200 and 400 mg/kg treated mice as compared with EtOH control mice, respectively(Table 3, Fig. 2).

Table 3. Changes on the Body Weight Gains

Groups	Body weights at administration			Weight gains [B-A]
	- 1 day of test material	First test material [A]	Last 14th test material [B]	
Controls				
Intact	22.16±1.20	20.11±1.19	26.29±1.46	6.18±0.82
EtOH	22.19±1.28	20.06±1.27	22.6±1.07^{A}	2.61±0.47^{A}
Silymarin 200 mg/kg	22.18±1.42	19.99±1.46	25.35±1.54^{C}	5.36±0.51^{BC}
GJD treated				
400 mg/kg	22.23±1.58	20.10±1.54	25.58±1.74^{C}	5.48±0.84^{C}
200 mg/kg	22.20±1.49	19.99±1.54	25.06±1.40^{C}	5.08±0.77^{AC}
100 mg/kg	22.15±1.20	19.78±1.38	24.68±1.17^{BC}	4.85±0.84^{AC}

Values are expressed as Mean±S.D. of eight mice, g. A $p<0.01$ and B $p<0.05$ as compared with intact control by LSD test C $p<0.01$ as compared with EtOH control by LSD test

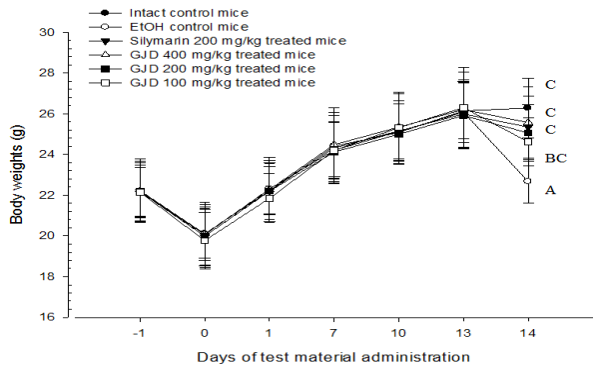

Fig. 2. Body Weight Changes in EtOH-treated Mice. Values are expressed as Mean±S.D. of eight mice, g or % of body weight p<0.01 as compared with intact control by LSD test B p<0.01 and C p<0.05 as compared with EtOH control by LSD test

2. Changes in the liver weight

Significant (p<0.01) decreases in the liver's absolute wet- and relative weight was detected in EtOH control mice as compared with Intact control mice. However, these EtOH-induced decreases in the liver absolute weight and relative weight was dramatically and significantly (p<0.01 or p<0.05) inhibited by treatment of silymarin 200 mg/kg and all three different dosages of GJD 400, 200 and 100 mg/kg as compared with EtOH control mice, in this experiment (Fig. 3).

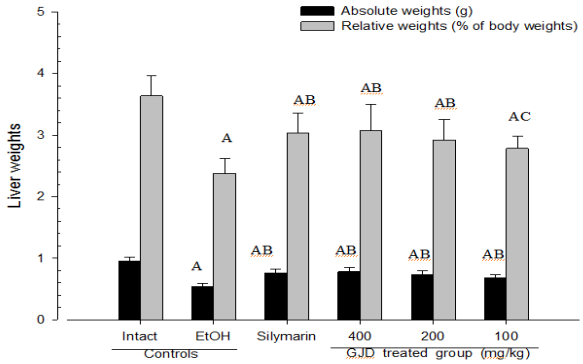

Fig. 3. Liver Weights Chages in EtOH-treated Mice. Values are expressed as Mean±S.D. of eight mice, g A p<0.01 and B p<0.05 as compared with intact control by LSD test C p<0.01 as compared with EtOH control by LSD test

3. Changes on the serum biochemistry, hepatic TG contents and TNF-α levels

Significant (p<0.01) increases of serum biochemistry, hepatic TG contents and TNF-α were observed in EtOH control as compared with Intact control. However, the serum biochemistry, hepatic TG contents and TNF-α levels significantly (p<0.01 or p<0.05) decreased by treatment of silymarin 200 mg/kg, GJD 400, 200 and 100 mg/kg in this experiment(Table 4, Fig. 4, Fig. 5).

Table 4. Changes on the Serum Biochemistry

Groups	erum biochemistrical levels				
	AST (IU/l)	ALT (IU/l)	Albumin (g/dl)	ALP (IU/l)	Triglyceride (mg/dl)
Controls					
Intact	99.25 ±19.35	49.75 ±10.15	3.76 ±0.66	59.20 ±14.46	127.00 ±21.00
EtOH	363.50 ±42.96^{C}	113.13 ±15.65^{A}	8.89 ±1.26^{A}	151.88 ±28.15^{A}	285.38 ±71.97^{C}
Silymarin 200 mg/kg	232.50 ±39.84^{CD}	73.50 ±11.29^{AB}	5.63 ±1.24^{AB}	87.25 ±14.95^{AB}	173.13 ±23.17^{CD}
GJD treated 400 mg/kg	223.25 ±43.23^{CD}	70.88 ±16.21^{AB}	5.36 ±1.03^{AB}	84.75 ±19.83^{AB}	161.00 ±16.93^{CD}
200 mg/kg	259.00 ±39.18^{CD}	81.38 ±5.97^{AB}	6.33 ±0.91^{AB}	103.75 ±12.85^{AB}	183.50 ±12.31^{CD}
100 mg/kg	283.75 ±20.63^{CD}	88.25 ±13.29^{AB}	7.06 ±1.05^{AB}	116.00 ±14.50^{AB}	205.00 ±16.47^{CD}

Values are expressed as Mean±S.D. of eight mice, g. A p<0.01 as compared with intact control by LSD test B p<0.01 as compared with EtOH control by LSD test C p<0.01 as compared with intact control by MW test D p<0.01 and E p<0.05 as compared with EtOH control by MW test.

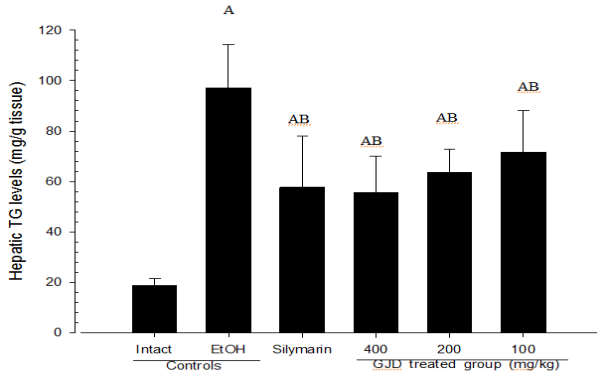

Fig. 4. Hepatic TG Content Changes in EtOH-treated Mice. Values are expressed as Mean±S.D. of eight mice, mg/g tissue A p<0.01 as compared with intact control by LSD test B p<0.01 as compared with EtOH control by LSD test.

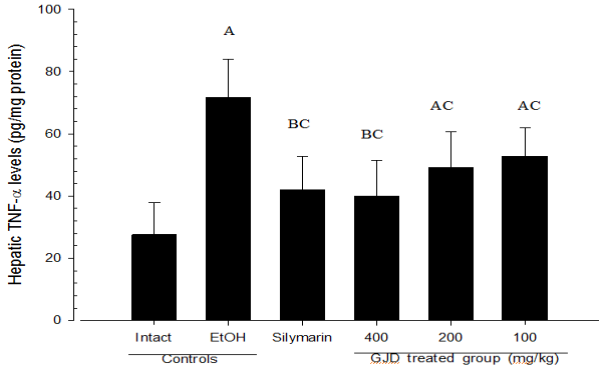

Fig. 5. Hepatic TNF-α Content Changes in EtOH-treated Mice. Values are expressed as Mean±S.D. of eight mice, pg/mg protein. A p<0.01 and B p<0.05 as compared with intact control by LSD test C p<0.01 as compared with EtOH control by LSD test.

4. Changes in the hepatic CYP 2E1 activity

Significant (p<0.01) increases in the liver's CYP 2E1 activity, hydroxylation of p-nitrophenol to 4-nitrocatechol, was demonstrated in EtOH control as compared with intact control mice. However, the liver CYP 2E1 activity

significantly(p<0.01) decreased by treatment of silymarin 200 mg/kg, GJD 400, 200 and 100 mg/kg, respectively(Fig. 6).

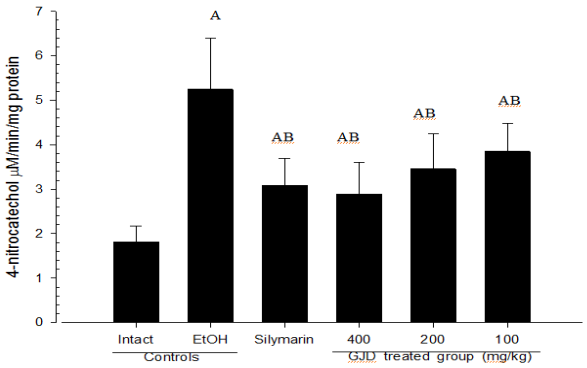

Fig. 6. Hepatic CYP 2E1 Activity Changes in EtOH-treated Mice. Values are expressed as Mean±S.D. of eight mice, 4-nitrocatechol μM/ min/mg protein. A p<0.01 as compared with intact control by MW test B p<0.01 as compared with EtOH control by MW test.

5. Changes in the hepatic antioxidant defense systems

Significant (p<0.01) increases of hepatic lipid peroxidation and MDA contents in liver parenchyma were observed in EtOH control mice as compared with Intact control mice. Significant (p<0.01) decreases of hepatic GSH contents, SOD and CAT activities were detected in EtOH control mice as compared with Intact control mice(Table 5).

Table 5. Hepatic Antioxidant Defense Systems in EtOH-treated Mice

Groups	MDA (nM/mg protein)	GSH (nM/mg protein)	SOD (U/mg protein)	CAT (U/mg protein)
Controls				
Intact	1.41±0.33	37.96±14.36	406.91±226.97	284.98±59.73
EtOH	3.88±0.71^{A}	9.70±1.72^{D}	173.85±42.23^{D}	118.86±25.48^{A}
Silymarin 200 mg/kg	2.27±0.42^{AB}	17.01±3.59^{DF}	279.51±44.48^{DF}	207.41±24.61^{AB}
GJD treated				
400 mg/kg	2.26±0.49^{AB}	17.03±2.02^{DF}	281.25±63.40^{EF}	208.45±40.26^{AB}
200 mg/kg	2.55±0.51^{AB}	15.80±2.76^{DF}	255.24±46.67^{DF}	189.75±46.45^{AB}
100 mg/kg	2.91±0.44^{AB}	13.87±3.33^{DG}	239.61±42.27^{DF}	163.26±28.48^{AC}

6. Changes in quantitative RT-PCR

To elucidate the molecular mechanism involved in the aggravation of ethanol-induced steatosis in GJD treated mice, the expression of genes regulating hepatic lipid synthesis was determined by quantitative RT-PCR, including SREBP-1c, SCD1, ACC1, FAS, PPARγ and DGAT2 and genes involved in fatty acid oxidation was also determined by quantitative RT-PCR, including PPARα, ACO and CPT1 in the present study.

1) Hepatic lipogenic genes expressions

Significant (p<0.01) decreases of the hepatic lipogenic genes -SREBP-1c, SCD1, ACC1, FAS, PPARγ and DGAT2 mRNA expressions were demonstrated in silymarin 200 mg/kg treated mice, and also dose-dependently in all three different dosages of GJD 400, 200 and 100 mg/kg treated mice as compared with EtOH control mice, in this experiment(Table 6).

2) Genes involved in fatty acid oxidation

Significant (p<0.01) increases of the hepatic PPARα, ACO and CPT1 mRNA expressions were demonstrated in GJD 400, 200 and 100 mg/kg treated mice with dose-dependent patterns, and also in silymarin 200 mg/kg treated mice as compared with EtOH control mice, in this experiment(Table 6).

Table 6. RT-PCR Analysis on the Hepatic Tissues

Groups	Controls		Silymarin	GJD treated		
Genes	Intact	EtOH	200 mg/kg	400 mg/kg	200 mg/kg	100 mg/kg
Hepatic lipogenic genes						
SREBP-1c	1.01±0.09	1.57±0.14^{A}	1.24±0.07^{AB}	1.22±0.06^{AB}	1.29±0.06^{AB}	1.35±0.09^{AB}
SCD1	0.99±0.07	2.43±0.33^{C}	1.66±0.21^{CD}	1.64±0.21^{CD}	1.85±0.10^{CD}	1.99±0.16^{CD}
ACC1	0.99±0.10	1.73±0.18^{A}	1.31±0.12^{AB}	1.31±0.14^{AB}	1.42±0.13^{AB}	1.49±0.11^{AB}
FAS	1.01±0.07	2.36±0.29^{A}	1.58±0.15^{AB}	1.58±0.16^{AB}	1.76±0.18^{AB}	1.91±0.17^{AB}
PPARγ	1.01±0.07	2.54±0.46^{C}	1.70±0.20^{CD}	1.69±0.22^{CD}	1.85±0.15^{CD}	2.01±0.15^{CD}
DGAT2	1.00±0.14	2.28±0.35^{C}	1.52±0.18^{CD}	1.51±0.29^{CD}	1.65±0.24^{CD}	1.81±0.12^{CD}
Genes involved in fatty acid oxidation						
PPARα	1.02±0.07	0.64±0.12^{C}	0.86±0.12^{CD}	0.87±0.06^{CD}	0.82±0.09^{CD}	0.78±0.05^{CE}
ACO	0.98±0.12	0.54±0.09^{A}	0.79±0.08^{AB}	0.79±0.09^{AB}	0.73±0.13^{AB}	0.69±0.08^{AB}
CPT1	1.06±0.09	0.53±0.09^{A}	0.79±0.09^{AB}	0.80±0.10^{AB}	0.75±0.08^{AB}	0.70±0.09^{AB}

Table 7. Hepatic Tissue Histopathological Analysis

Index / Groups	Fatty change regions (%)	Fatty changed hepatocyte numbers (/1000 cells)	Mean hepatocyte diameters (μm)	Numbers NT-immunol abeled cells (/1000 cells)	Numbers 4-HNE-immun opositive cells (/1000 cells)
Controls					
Intact	7.72 ±2.84	81.88 ±48.29	21.37 ±5.28	53.63 ±36.64	67.88 ±33.80
EtOH	72.05 ±13.39^{A}	695.13 ±147.66^{A}	33.24 ±3.80^{C}	318.25 ±97.05^{C}	527.00 ±142.81^{CE}
Silymarin 200 mg/kg	32.26 ±13.02^{AB}	317.00 ±141.63^{AB}	26.15 ±2.77^{E}	160.88 ±31.67^{CE}	265.00 ±105.77^{CE}
GJD treated					
400 mg/kg	32.32 ±10.07^{AB}	314.75 ±95.24^{AB}	25.79 ±3.09^{E}	157.63 ±23.42^{CE}	259.25 ±61.23^{CE}
200 mg/kg	42.86 ±11.04^{AB}	428.25 ±112.65^{AB}	27.11 ±2.82^{DE}	186.63 ±19.43^{CE}	305.38 ±91.51^{CE}
100 mg/kg	53.61 ±11.16^{AB}	498.63 ±104.28^{AB}	28.52 ±2.26^{CF}	208.50 ±34.59^{CE}	336.75 ±82.87^{CE}

7. Effects on the liver histopathology

Severe deposition of lipid droplets in cytoplasm of hepatocytes, hepatosteatosis were observed in all EtOH-dosing groups in the present study, and these EtOH-induced hepatosteatosis are re-confirmed with histomorphometry as the numbers of fatty changed hepatocytes, mean diameters of hepatocytes and percentages of fatty changed regions, which were significantly (p<0.01) increased in EtOH control mice as compared with Intact control mice, respectively. However, these EtOH treatment-related histopathological hepatosteatosis were significantly (p<0.01 or p<0.05) and

dose-dependently inhibited by treatment of all three different dosages of GJD 400, 200 and 100 mg/kg as compared with EtOH control mice, and also significantly ($p<0.01$) by treatment of silymarin 200 mg/kg as compared with EtOH control mice, in this experiment(Fig. 7, Table 7).

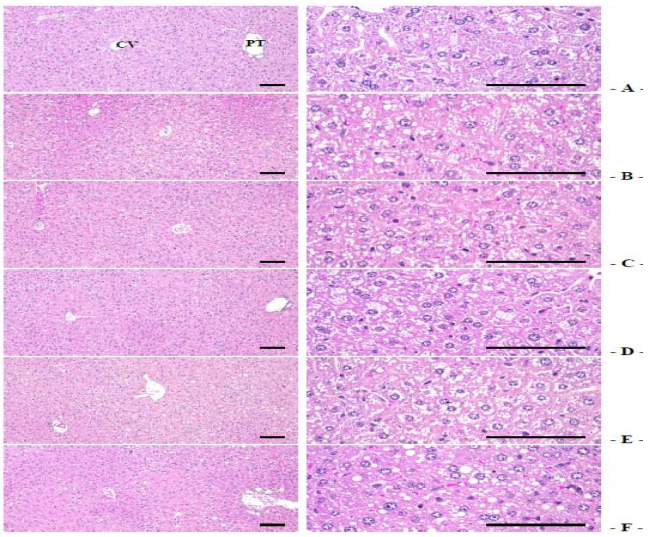

Fig. 7. Representative Histological Liver Images. A = Intact control mice. B = EtOH control mice. C = Silymarin 200 mg/kg treated mice. D = GJD 400 mg/kg treated mice. E = GJD 200 mg/kg treated mice. F = GJD 100 mg/kg treated mice. Hematoxylin-eosin stain Scale bars = 200 μm

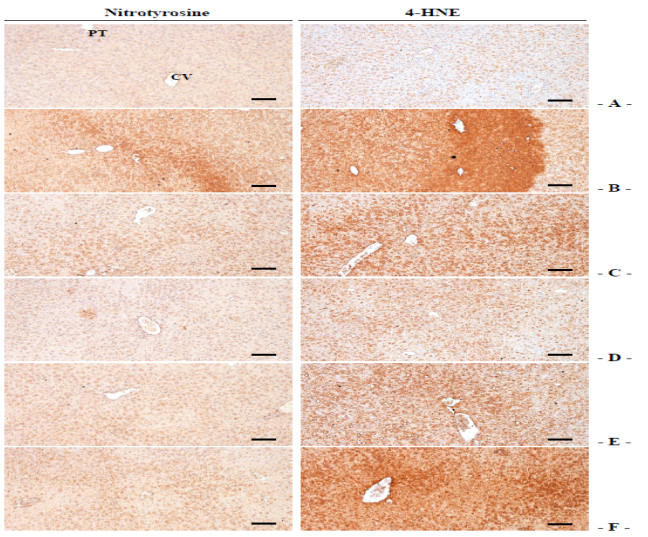

Fig. 8. Representative Images of Nitrotryrosine and 4-HNE-immunoreactivities in the Liver Sections. A = Intact control mice. B = EtOH control mice. C = Silymarin 200mg/kg treated mice. D = GJD 400mg/kg treated mice. E = GJD 200mg/kg treated mice. F = GJD 100mg/kg treated mice. Scale bars = 200 μm.

8. Effects on the immunohistochemistry

The immunoreactivities of NT as marker of iNOS related oxidative stresses[23] and 4-HNE as marker of lipid peroxidation[24] in hepatic parenchyma were assessed to observe the liver oxidative stresses, respectively. Marked and significant ($p<0.01$) increases of an iNOS related oxidative stress marker, NT-immunoreactive hepatocytes were observed in EtOH control mice as compared with intact control mice. Lipid peroxidation marker, 4-HNE-immunoreactive hepatocytes were also significantly ($p<0.01$) increased in EtOH control mice as compared with Intact control mice(Fig. 8, Table 7).

Discussion

Gongjin-dan is a famous traditional Korean tonifying polyherbal prescription and has shown hypolipidemic activities[8]. Also the hepatoprotective effects of Gongjin-dan in alcoholic liver damage in rats already has been reported by other investigators[9]. Therefore we intended to observe the potential hepatoprotective effects of Gongjin-dan on the acute EtOH-induced hepatic damages in C57BL/6 mice through its anti-inflammatory, anti-oxidant and anti-steatosis mechanisms.

After EtOH treatment, decrease in body weight was considered as the results of direct toxicity of EtOH, indirect toxicity related to the hepatic damages. Therefore, the increased body weight and gains are considered indirect evidences that they have liver-protective effects on the EtOH-induced hepatic damages, since weight is considered as a putative indicator of health. Inhibitory effects, in addition, on the EtOH-induced liver weight decreases by silymarin 200 mg/kg treatment, and also dose-dependently by treatment of GJD 400, 200 and 100 mg/kg were also considered as truthful evidences that GJD has hepatoprotective effects against acute EtOH intoxications.

Generally AST, ALT, ALP, albumin used as serum markers which represented the liver damages, and markedly elevated in EtOH-induced liver damages in previous reports[25,26] and also in this experiment. Serum TG levels are increased generally as a process of EtOH-induced liver damages due to TG utilizations' decreases in hepatocytes[27]. Therefore as direct evidences, it is considered that GJD 400, 200 and 100 mg/kg, silymarin 200 mg/kg have hepatoprotective effects against EtOH-induced hepatic injuries. Especially GJD showed obvious dose-dependent inhibition on EtOH-induced serum AST, ALT, albumin, ALP and TG level changes, and also on the hepatic TG contents compared with EtOH control mice in this experiment.

Abnormal metabolism of cytokine, especially TNF-α, is another major feature of alcoholic liver disease[6]. This study showed that acute EtOH administration enhanced hepatic TNF-α production, and in vivo GJD 400, 200 and 100 mg/kg

administration dose-dependently attenuated this increased TNF-α production, respectively.

Long-term alcohol exposure increased CYP 2E1 activities[28]. Furthermore investigations using CYP 2E1 inhibitors have shown inhibition of CYP 2E1 activity reduced alcohol-induced liver injury, indicating the importance of CYP 2E1 in alcohol-induced ROS accumulation and liver injury[29]. To investigate the possible mechanisms by which GJD attenuated acute EtOH-induced liver injury, first we evaluated the effect of GJD on CYP 2E1 enzymatic activity in acute EtOH exposure.

The metabolism of ethanol gives rise to the generation of excess amounts of ROS and has a detrimental effect on cellular antioxidant defense system[30] leads to hepatic cellular necrosis, inflammation and steatohepatitis[3,4]. Oxidative destruction of cellular membranes is able to lead to cell death and to production of reactive aldehyde and toxic metabolites called free radicals, which MDA is the most important[31]. MDA is a lipid peroxidation's terminal product. GSH is endogenous representative antioxidants and it prevent tissue damage by keeping the ROS at low levels, certain cellular concentrations. SOD is antioxidant enzymes that served to enzymatic defense mechanisms. The decrease of anti-oxidant enzyme (such as SOD and catalase, GSH contents) activities may be indicative of the failure of redress the induced oxidative stress induced by EtOH[3,30]. In this experiment, the liver antioxidant defense systems were dose-dependently enhanced by treatment of GJD 400, 200 and 100 mg/kg as compared with EtOH control, respectively.

Previous studies demonstrated that EtOH administration activates SREBP-1c and its target genes like ACC1, FAS, and SCD1 which promote de novo fatty-acid synthesis[5,32]. In this experiment, EtOH treatment up-regulated significantly the hepatic mRNA expression of SREBP-1c and its target genes – ACC1, FAS, and SCD1 respectively. However, GJD 400, 200 and 100 mg/kg down regulated the liver mRNA expression of SREBP-1c, SCD1, ACC1 and FAS, suggesting hepatoprotective effects of GJD against EtOH-induced liver steatosis are mediated by down regulation of SREBP-1c and its target genes, ACC1, FAS, and SCD1, partially in this experiment. GJD 400 mg/kg down regulated liver mRNA expression of SREBP-1c and its target genes, as similar to those of silymarin 200 mg/kg, in the present study.

PPARγ and DGAT are significantly up-regulated after acute EtOH administration and involved in ethanol-induced fatty liver in mouse[5,32]. In this study, hepatic mRNA levels of the both PPARγ and DGAT2 were all up-regulated by EtOH stimulation, but all three different dosages of GJD 400, 200 and 100 mg/kg significantly impaired elevations of these genes. These results suggested that oral treatment of GJD 400, 200 and 100 mg/kg inhibits liver lipogenesis responsed to EtOH by suppressing genes, at least, related to TG synthesis partially in this experiment. Acute binge treatment of EtOH, in this experiment, decreased expression of PPARα, CPT1 and ACO, impaired fatty-acid β-oxidation in the liver.

Acute or chronic alcohol consumption cause severe histopathological liver injury[33]. Severe deposition, in this experiment, of lipid droplets in cytoplasm of hepatosteatosis, hepatocytes were also observed in all EtOH treated mice. These EtOH-induced hepatosteatosis are reconfirmed as histo-morphometry of the numbers of fatty changed mean diameters of hepatocytes, percentages of fatty changed regions, hepatocytes which were increased significantly in EtOH control mice as compared with Intact control mice respectively in the left lateral lobes.

NT is tyrosine nitration product mediated by reactive nitrogen species such as nitrogen dioxide and peroxynitrite anion. Sustained exposure to ROS prolonged oxidative stress, increases of NT[34]. In our experiment, significant and marked increases of NT immuno-reactive cells were observed in hepatic tissues of EtOH control mice but they were significantly and dose-dependently reduced by treatment of GJD 400, 200 and 100 mg/kg. It means that 400, 200 and 100 mg/kg of GJD favorably inhibited iNOS related oxidative stresses and protect hepatocyte necrotic changes from EtOH.

4-HNE is produced by lipid peroxidation in cells, which is an α, β-unsaturated hydroxyalkenal. Sustained exposure to ethanol-mediated ROS leads to oxidative stress, which promotes lipid generation and peroxidation of reactive aldehydes, such as 4-HNE[34,35]. In this study, the results of NT-immunolabeled cells considered as direct evidences that they were inhibited effectively lipid peroxidations, the formation of 4-HNE to protect necrotic changes from EtOH in the model used in the current study.

Conclusion

In this study, the results suggest that oral administration of 400, 200 and 100 mg/kg of GJD protected favorably the liver damages from acute mouse EtOH intoxications, and mediated by its potent anti-inflammatory, anti-steatosis proprieties through augmentation of liver antioxidant defense system and up-regulation of mRNA expressions of genes involved in fatty acid oxidation or

down-regulation of mRNA expressions of hepatic lipogenic genes.

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