

Original Article

***Kyungheechunggan-tang* suppresses inflammatory cytokines and fibrotic genes in LPS-induced RAW 264.7 cells and LX-2 cells**

Junghan Bae, Eungyeong Jang, Jang-Hoon Lee

Department of Internal Medicine, College of Korean Medicine, Kyung Hee University

Objectives: The aim of this study is to investigate anti-inflammatory effects of *Kyungheechunggan-tang* (KHC GT) on LPS- induced RAW 264.7 cells and LX-2 cells and anti-fibrotic effects of KHC GT on LX-2 cells.

Materials and Methods: Three types of KHC GTs (KHC GT-A, -B, and -C) by narrowing down the number of constituent herbs from 9 (KHC GT-A) to 5 (KHC GT-B) and to 3 (KHC GT-C) were developed. To understand pharmacological effects of KHC GT, three types of KHC GTs were treated on RAW 264.7 cells and LX-2 cells. Anti-inflammatory activities of KHC GT were evaluated by ELISA assay for pro-inflammatory cytokines, IL-6, TNF- α and IL-10, in LPS-stimulated RAW 264.7 cells and for IL-6 production in LPS-induced LX-2 cells. In addition, anti-fibrotic effects of KHC GT were determined by quantitative real-time PCR assay for fibrosis-related genes, α -SMA, collagen1A1, TIMP1, MMP-2, in LX-2 cells.

Results: KHC GT-A and KHC GT-C showed inhibitory effects on secretion of IL-6 in LPS-stimulated RAW 264.7 cells and LX-2 cells. KHC GT-B and KHC GT-C exhibited inhibitory effects on the expression of pro-inflammatory cytokines such as IL-6, TNF- α , and IL-10 in LPS-stimulated RAW 264.7 cells. The mRNA expression levels of collagen1A1 and MMP-2 were significantly reduced by KHC GT-C whereas TIMP-1 was suppressed by KHC GT-A and KHC GT-B in LX-2 cells. Among three different formulas, KHC GT-C demonstrated the most remarkable effects on inflammation and fibrosis.

Conclusions: In this study, KHC GT showed both anti-inflammatory and anti-fibrotic effects which make it to be a prospective agent for chronic liver diseases with inflammation and fibrosis.

Key Words : *Kyungheechunggan-tang*, RAW 264.7, LX-2, Inflammation, Fibrosis

Introduction

Inflammation is an immune response that protects host from harmful stimulants such as infection, cellular damage, and tissue injury¹⁻²). The inflammatory response induces activation and recruitment of immune cells, resulting in production of a variety of inflammatory mediators³). In particular, pro-inflammatory cytokines

such as tumor necrosis factor (TNF)- α and interleukin (IL)-6 were often observed in the pathogenesis of chronic liver inflammation⁴). Moreover, the etiology of liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) is also related to inflammatory pathology, showing the elevation of TNF- α and IL-6⁵⁻⁷).

Hepatic fibrosis, a healing process for liver damage, is the result driven by inflammation responses in liver.

• Received : 16 April 2018 • Revised : 8 May 2018 • Accepted : 8 May 2018

• Correspondence to : Jang-Hoon Lee

Department of Hepato-hemopoietic System, Kyung Hee University Korean Medicine Hospital, 23, Kyung-Hee-dae-ro, Dongdaemun-gu, Seoul 02447, Republic of Korea

Tel : +82-2-958-9118, Fax : +82-2-958-9258, E-mail : komclive@khmc.or.kr

As a result of the inflammation process, macrophage-derived transforming growth factor (TGF)- β 1 stimulates the activation of hepatic stellate cells (HSCs)⁸⁻⁹. Activated HSCs result in excessive accumulation of the extracellular matrix (ECM) including α -smooth muscle actin (SMA), type I collagen (collagen I), matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs)¹⁰⁻¹¹. Liver fibrosis is a reversible state, but the possibility of recovery is substantially reduced if it progresses to cirrhosis or HCC¹²⁻¹⁴. Although efficient therapeutic strategies are urgently required, until now there is no standard treatment for ameliorating liver inflammation and fibrosis simultaneously¹⁵⁻¹⁷.

IL-6 and TNF- α are pro-inflammatory cytokines mediating inflammatory responses and are secreted in macrophages and Kupffer cells^{6,18}. IL-10 is a cytokine secreted by macrophage activation and liver injury processes which can intervene pro-inflammatory macrophage function. Collagen I and α -SMA, the most

abundant factors of ECM components, are mainly produced in activated stellate cells and are responsible for cell structure, motility and contractility^{9,19}. MMP-2 is also expressed in activated stellate cell, related to HSCs proliferation²⁰ and regulated by TIMPs²¹. TIMP1 is an inhibitor of activated HSCs-apoptosis and appears to be an important factor in promoting hepatic fibrosis^{10,22-23}.

Kyungheechunggan-tang (KHCGT)-A is composed of 9 kinds of herbs (Table 1) which was used for the treatment of acute/chronic liver diseases²⁴. In the previous studies, KHCGT-A was demonstrated to show hepato-protective effects by reducing cellular steatosis, reactive oxygen species (ROS) production, and apoptosis²⁴ and anti-fibrotic effects by suppressing collagen I and α -SMA mRNA expression⁹. The most important components among herbs composed of KHCGT-A are thought to be *Artemisia capillaris* (AC), *Alisma orientale* (AO), and *Curcuma longa* (CL). AC has been represented to exhibit anti-inflammatory²⁵

Table 1. Composition of Three Herbal Formulas of KHCGT

	Scientific name	Pharmacognostic name	Weight (g)
KHCGT-A	<i>Artemisia capillaris</i>	Artemisiae Capillaris Herba	30
	<i>Alisma orientale</i>	Alismatis Rhizoma	15
	<i>Atractylodes japonica</i>	Atractylodis Rhizoma Alba	12
	<i>Salvia miltiorrhiza</i>	Salviae Miltiorrhizae Radix	8
	<i>Curcuma longa</i>	Curcuma Radix	8
	<i>Crataegus pinnatifida</i>	Crataegi Fructus	8
	<i>Lycium chinense</i>	Lycii Fructus	8
	<i>Poncirus trifoliata</i>	Aurantii Immaturus Fructus	6
	<i>Glycyrrhiza uralensis</i>	Glycyrrhizae Radix	6
Total			101
KHCGT-B	<i>Artemisia capillaris</i>	Artemisiae Capillaris Herba	45
	<i>Alisma orientale</i>	Alismatis Rhizoma	15
	<i>Curcuma longa</i>	Curcuma Radix	12
	<i>Crataegus pinnatifida</i>	Crataegi Fructus	12
	<i>Lycium chinense</i>	Lycii Fructus	12
Total			96
KHCGT-C	<i>Artemisia capillaris</i>	Artemisiae Capillaris Herba	45
	<i>Alisma orientale</i>	Alismatis Rhizoma	15
	<i>Curcuma longa</i>	Curcuma Radix	15
Total			75

and anti-fibrotic effects²⁶⁾ and to have the most significant anti-fibrotic effect among the KHCGT-A constitutive agents⁹⁾. AO has been implicated in the protection against inflammation and fibrosis on non-alcoholic fatty liver disease (NAFLD)²⁷⁾, and CL also has been demonstrated to have anti-inflammatory²⁸⁾ and anti-fibrotic effects⁹⁾. In addition, hepato-protective agents *Crataegus pinnatifida* (CP) and *Lycium chinense* (LC) were also considered to be important, because CP has shown anti-inflammatory potentials *in vitro* and *in vivo* against lipopolysaccharide (LPS) treatment²⁹⁾ and LC has been reported to show anti-inflammatory³⁰⁾ and anti-fibrotic effects³¹⁾ against hepatic disorders. Based on these herbs, KHCGT-B (AC, AO, CL, CP, and LC) and KHCGT-C (AC, AO, and CL), were newly developed.

The aim of this study is to confirm the anti-inflammatory and anti-fibrotic effects of KHCGTs (KHCGT-A, -B, and -C) on RAW 264.7 cells and LX-2 cells. In order to evaluate their anti-inflammatory effects, RAW 264.7 cells and LX-2 cells were induced by LPS followed by KHCGTs treatment and for the purpose of observing the anti-fibrotic effects, KHCGTs were applied to LX-2 cells.

Materials and methods

1. Preparation of herbal extracts

All herbal plants consisting of *Kyungheechunggan-tang* (KHCGT) (Table 1) were purchased from Kyung Hee Herb Pharm (Wonju, South Korea), an herbal company operated by Korea Good Manufacturing Practice (KGMP) guideline. KHCGT-A 101 g, KHCGT-B 96 g, and KHCGT-C 75 g were extracted for 3 h with 30 % ethanol of 1000 ml, 1000 ml, and 750 ml, respectively. Each extracted solutions were filtrated, followed by the repeated extraction process once more. Herbal samples were then evaporated,

freeze-dried in order. After processing in freeze dryer apparatus, a form of dried extract powders of each herbal decoction was obtained as 33.72 g of KHCGT-A, 25.38 g of KHCGT-B and 14.40 g of KHCGT-C with extraction yield of 33.39 %, 26.44 % and 19.20 %, respectively.

2. Cell culture

2.1. LX-2 cells

LX-2 cells, immortalized human hepatic stellate cells, were kindly provided by Professor Sang Geon Kim (College of Pharmacy, Seoul National University, Seoul, South Korea). LX-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, MA, USA) supplemented with 10 % fetal bovine serum (FBS; Equitech-bio, Texas, USA) and 1 % antibiotics (Hyclone, MA, USA) in an atmosphere of 5 % CO₂ at 37 °C.

2.2. RAW 264.7 cells

RAW 264.7 mouse monocyte cells were obtained from the Korean Cell Line Bank (Seoul, South Korea). These murine macrophage cells were grown in DMEM media with 10 % of FBS and 100 U/mL of antibiotics, and cultured at 37 °C with humidified air of 5 % CO₂.

3. Lipopolysaccharide (LPS) and herbal treatment

RAW 264.7 cells and LX-2 cells were pretreated with or without three different herbal extracts (KHCGT-A, -B, and -C) for 24 h, and challenged with LPS (Sigma, St Louis, MO, USA) 50 ng/ml and 100 ng/ml treatment for 18 h and 4 h, respectively.

4. Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium

bromide (MTT) assay (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. LX-2 cells and RAW 264.7 cells were seeded in 96-well plates at a density of 5×10^3 cells and 1.1×10^4 cells per well in DMEM media, respectively. After 24 h of stabilization, three kinds of herbal samples were applied with various concentrations (0, 7, 15, 31, 62, 125, 250, 500 $\mu\text{g/ml}$) and the cells were incubated for another 24 h and 48 h. Subsequently, 100 μl of MTT solution (Promega, Madison, WI, USA) was added for 2 h, and the cells were successively incubated for another 2 h at 37 $^{\circ}\text{C}$ in a humid atmosphere of 5 % CO_2 . The insoluble formazan crystals were dissolved in 150 $\mu\text{l/well}$ dimethylsulfoxide (DMSO; Lonza, Allendale, NJ, USA) and absorbance value was read at 540 nm by a microplate reader (BMG LABTECH, Ortenberg, Germany).

5. ELISA assay for IL-6, TNF- α , and IL-10

RAW 264.7 cells and LX-2 cells were plated in 24 well plates at a density of 2.5×10^4 cells per well in 500 μl of DMEM media containing 10 % FBS and 100 U/ml of antibiotics for 24 h. After overnight acclimation, the cells were pretreated with 125 and 250 $\mu\text{g/ml}$ of herbal medicine samples for another 24 h, and then stimulated with LPS 50 ng/ml for 18 h and 100 ng/ml for 4 h for RAW 264.7 cells and LX-2 cells, respectively. The supernatants from each well were collected and centrifuged at 12000 rpm and 25 $^{\circ}\text{C}$ for 1 min. The mouse ELISA kit (OptEIA mouse IL-6, TNF- α , IL-10 ELISA set and human IL-6 set, BD, CA, USA) was used for the quantitative

determination of IL-6, TNF- α , and IL-10 levels contained in the supernatants, according to the manufacturer's protocol. After treating the stop solution, the quantitative expression amounts of IL-6, TNF- α , and IL-10 were measured at 450 nm by a microplate reader (BMG LABTECH, Ortenberg, Germany).

6. Quantitative real-time PCR analysis

Total cellular RNA was extracted from the LX-2 cells using the Hybrid-R kits (GeneAll, Korea), and isolated RNA was reverse-transcribed using RT dryMIX (Enzynomics, Korea). The purity and quantity of RNA samples were determined by Nanodrop 2000 (Thermo Scientific). Quantitative real-time PCR was performed using CFX96 TouchTM Real-Time PCR Detection System (BIORAD, USA) which was set up with 40 cycles.

- (1) Collagen1A1 and α -SMA: First cycle at 95 $^{\circ}\text{C}$ for 3 min, 40 cycles at 95 $^{\circ}\text{C}$ for 15 sec, 59 $^{\circ}\text{C}$ for 10 sec, 72 $^{\circ}\text{C}$ for 40 sec, and last cycle at 95 $^{\circ}\text{C}$ for 10 sec
- (2) TIMP1 and MMP-2: First cycle at 95 $^{\circ}\text{C}$ for 3 min, 40 cycles at 95 $^{\circ}\text{C}$ for 15 sec, 61.4 $^{\circ}\text{C}$ for 10 sec, 72 $^{\circ}\text{C}$ for 40 sec, and last cycle at 95 $^{\circ}\text{C}$ for 10 sec

The primers used for complementary DNA amplification of α -SMA, Collagen1A1, TIMP1, MMP-2, and β -actin genes are presented in Table 2. The mRNA expression results of each gene were normalized with β -actin mRNA level.

Table 2. Primer Sequences for Real-Time PCR

Genes	Sense primer	Anti-sense primer
α -SMA	TGTGAATGTCCTGTGGAATTATGC	ACACATAGGTAACGAGTCAGAGC
Collagen1A1	CAGCCGCTTCACCTACAGC	TTTGTATTCAATCACTGCTTGCC
TIMP1	GCCCAGAGAGACACCAGAGAAC	CTATCAGCCACAGCAACAACAGG
MMP-2	TGACGGTAAGGACGGACTC	ATACTTCACACGGACCACTTG
β -actin	GGGAAATCGTGCGTGCATT	GGAGTTGAAGGTAGTTTCGT

α -SMA: α -smooth muscle actin; TIMP1: tissue inhibitors of metalloproteinases 1; MMP-2: matrix metalloproteinases-2

7. Statistical analysis

All values of the experimental data were expressed as mean \pm SD. One-way ANOVA and LSD's post-hoc tests were performed to compare the results and assess the statistical significance of differences between groups. *p* values less than 0.5 were defined as significant.

Results

1. Effects of KHC GT on cell viability

To clarify safeness of KHC GT on LX-2 cells and RAW 264.7 cells, MTT assay on both cells was performed with all three kinds of KHC GTs (KHC GT-A, -B and -C) for 24 h and 48 h, respectively. Treatment on LX-2 cells of KHC GTs with different concentrations (0, 7, 15, 31, 62, 125, 250, 500 μ g/ml) exhibited no significant decrease in cell viability in all three types of KHC GTs after overnight and 2 days (Figs. 1A and

1B), suggesting that herbal samples affect no direct toxic changes on HSCs. Similarly, all sorts of KHC GTs at the same concentrations did not cause cytotoxicity in RAW 264.7 cells for 24 h and 48 h (Figs. 1C and 1D). The 50 % cytotoxic concentration (CC_{50}) values were estimated more than 500 μ g/ml (Fig. 1). These data indicate that three KHC GT formula samples have no cytotoxicity on both LX-2 cells and RAW 264.7 cells.

2. Effects of KHC GT on inflammatory mediators in LPS-stimulated RAW 264.7 cells

To determine anti-inflammatory effects of KHC GT extracts, the productions of IL-6, TNF- α , and IL-10 in LPS-stimulated RAW 264.7 cells were evaluated by ELISA. In response to LPS 50 ng/ml treatment, RAW 264.7 cells expressed increased cytokine release

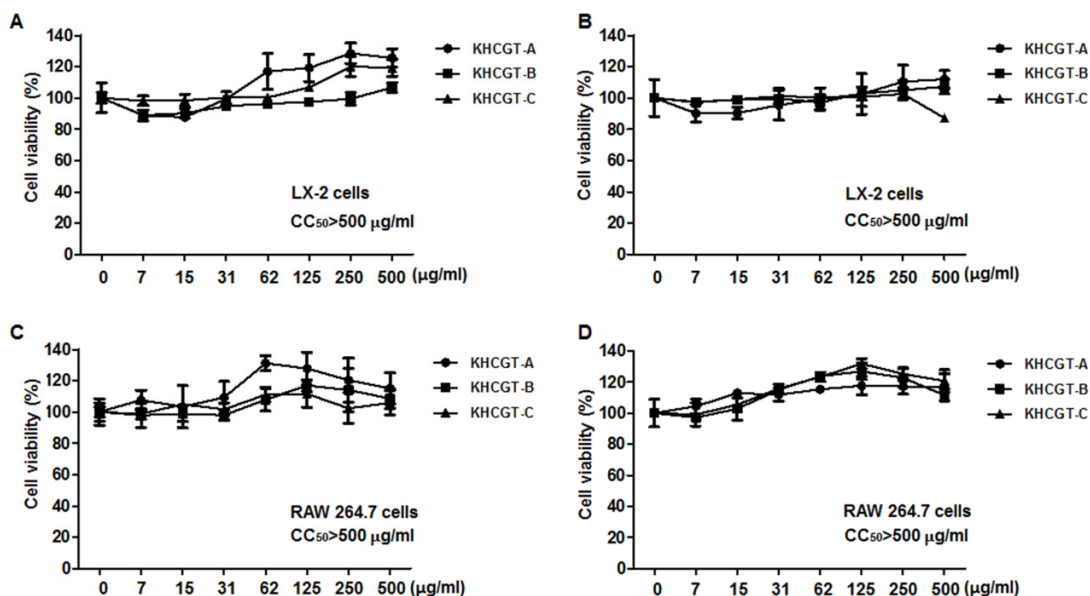


Fig. 1. Cell viability assay

Effects of KHC GT-A, -B, and -C on cell viability of LX-2 cells for 24 h (A) and 48 h (B), and RAW 264.7 cells for 24 h (C) and 48 h (D). Cells were treated with various concentrations of three kinds of KHC GTs (0–500 μ g/ml). Cell viability was measured by MTT assay. Data were expressed as mean \pm SD from three separate experiments.

and these pro-inflammatory cytokines have been reported to contribute to a variety of inflammation-related diseases. IL-6 level was significantly reduced after 24 h pre-treatment with KHC GT-A, -B, and -C in a concentration-dependent manner. Particularly, KHC GT-C treatment showed the most potent inhibitory effect among three herbal formulas against IL-6 secretion, exhibiting low detection of IL-6 expression similar to the control group without LPS treatment (Fig. 2A). As shown in Figs. 2B and 2C, KHC GT-B and -C showed significant suppression on TNF- α level compared to LPS-treated RAW 264.7 cells. In accordance with the effects of KHC GT-C on IL-6 production, 250 μ g/ml of KHC GT-C revealed the most

marked inhibitory effects on TNF- α and IL-10 levels among three samples in a dose-dependent manner ($p < 0.001$).

3. Effects of KHC GT on IL-6 production in LPS-induced LX-2 cells

It is known that LPS-induced upregulation of IL-6 is involved in HSCs activation, a critical initial signal in the pathogenesis of liver fibrosis⁸). To examine whether KHC GT-A, -B, and -C inhibit IL-6 levels produced in response to LPS stimulants on LX-2 cells, ELISA was performed with the supernatants of these formula-treated cells. LPS treatment of 100 ng/ml for

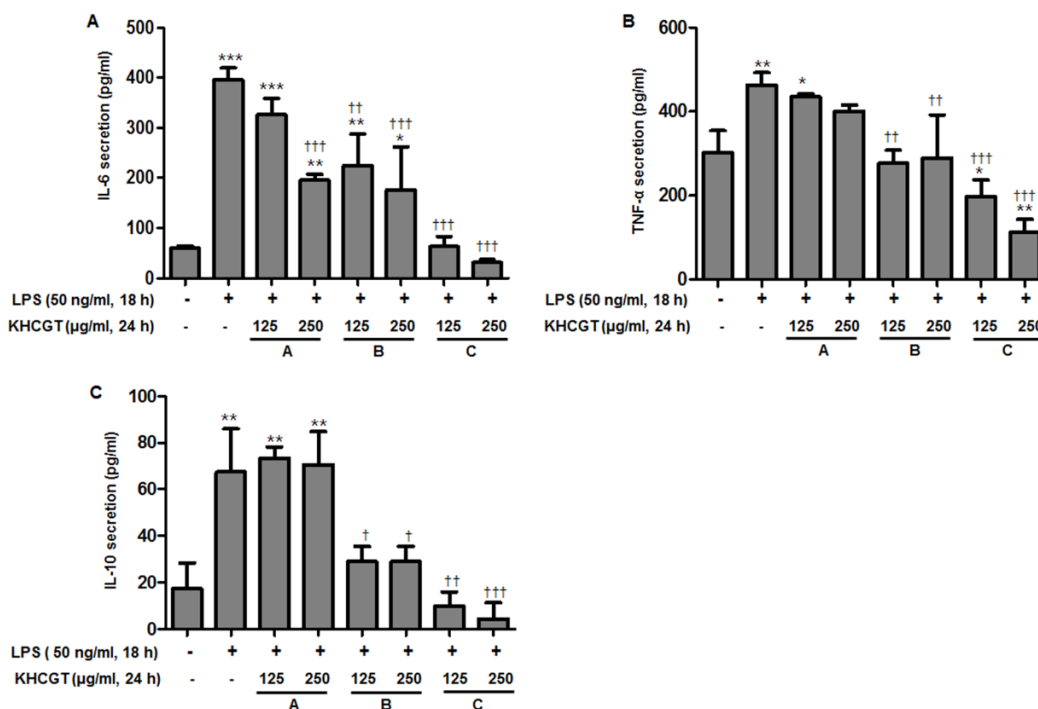


Fig. 2. ELISA assay for pro-inflammatory cytokines

Effects of KHC GT-A, -B, and -C on IL-6 (A), TNF- α (B), and IL-10 (C) secretion in LPS-stimulated RAW 264.7 cells. Cells were treated with three kinds of KHC GT-A, -B, and -C of 125 and 250 μ g/ml. Data were derived from three independent experiments and presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the control group without LPS or KHC GT. † $p < 0.05$, †† $p < 0.01$, and ††† $p < 0.001$ compared to the LPS-stimulated group.

4 h on LX-2 cells triggered significant secretion of IL-6 ($p < 0.01$). As shown in Fig. 3, 125 $\mu\text{g/ml}$ of KHCGT-A and 250 $\mu\text{g/ml}$ of KHCGT-C significantly inhibited the increased level of IL-6 ($p < 0.05$).

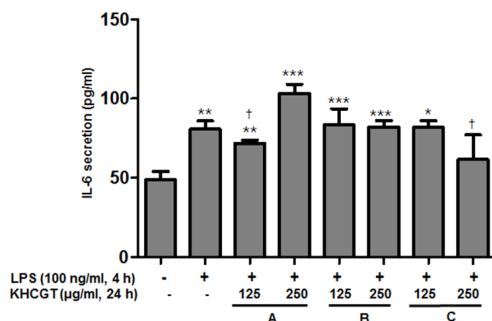


Fig. 3. ELISA assay for IL-6 production

Effects of KHCGT-A, -B, and -C on IL-6 secretion in LPS-induced LX-2 cells. Cells were treated with three kinds of KHCGT-A, -B, and -C of 125 and 250 $\mu\text{g/ml}$. Data were derived from three independent experiments and presented as mean \pm SD. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to the control group without LPS or KHCGT. † $p < 0.05$, compared to the LPS-stimulated group.

4. Effect of KHCGT on fibrosis-related genes in LX-2 cells

Real-time PCR was performed to evaluate the inhibitory effects of KHCGT-A, -B, and -C on the mRNA levels of α -SMA, collagen1A1, TIMP1, and MMP-2 in LX-2 cells. Each herbal extract was applied to cells at 250 $\mu\text{g/ml}$ which concentration was shown to have significant inhibitory effects on pro-inflammatory mediators (Fig. 2). As illustrated in Fig. 4, KHCGT-C 250 $\mu\text{g/ml}$ significantly inhibited the mRNA expressions of collagen1A1 and MMP-2 ($p < 0.05$, Figs. 4B and 4D), but there was no significant changes on α -SMA and TIMP1 mRNA levels (Figs. 4A and 4C). Regarding TIMP1 mRNA levels, KHCGT-A and -B showed significant reduction on relative mRNA expression compared to the control group ($p < 0.05$, Fig. 4C).

Discussion and conclusion

Inflammation and fibrosis play crucial roles in determining liver damage in a wide range of chronic liver diseases, including nonalcoholic fatty liver diseases (NAFLD), viral hepatitis, and alcoholic liver diseases³²). Inflammation-driven pathological process in liver activates quiescent HSCs which leads to accumulation of collagen I and α -SMA¹¹). Inversely, stimulated HSCs are often found to produce pro-inflammatory cytokines that keep themselves activated or contribute to the progression to severe fibrosis³³). Overall, targeting both inflammation and fibrosis might be of special difficulty but potent therapeutic strategy for treating chronic liver diseases, and numerous herbal medicines such as *Saururus chinensis*, *Salvia miltiorrhiza*, and *Scutellaria baicalensis* have been shown to have inhibitory effects on both inflammation and fibrosis³⁴).

KHCGT-A is a Korean herbal formula composed of 9 medicinal herbs. The Monarch and Minister drug of this decoction is AC and AO, respectively. Besides these two main drugs, KHCGT consists of *Atractylodes japonica* (AJ), *Salvia miltiorrhiza* (SM), CL, CP, LC, *Poncirus trifoliata* (PT) and *Glycyrrhiza uralensis* (GU). In a previous study, 100 $\mu\text{g/ml}$ water extract of KHCGT-A inhibited steatosis and ROS production by regulating apoptosis in palmitate-induced HepG2 cell models²⁴). In addition, KHCGT-A showed anti-fibrotic effects against activated LX-2 cells and dimethylnitrosamine-induced rat liver by downregulating mRNA levels of collagen I and α -SMA⁹). KHCGT-A might exert anti-inflammatory activities because AC and AO, main drugs of KHCGT-A, have been reported to regulate nitric oxide, NF- κ B, COX-2, prostaglandin E2, and TNF- α ^{25,35-36}). However, none of the previous studies demonstrated anti-inflammatory effects of KHCGT-A. Furthermore, there is no precedent study

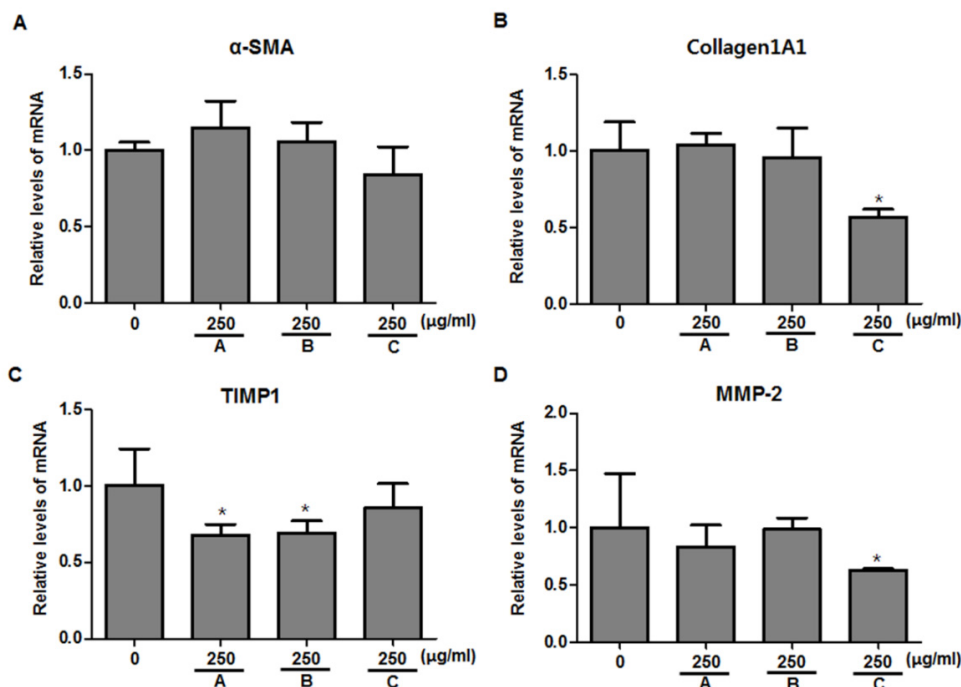


Fig. 4. Real-time PCR assay to determine fibrosis-related genes. Effects of KHC GT-A, -B, and -C on α -SMA (A), collagen1A1 (B), TIMP1 (C), and MMP-2 (D) mRNA levels in LX-2 cells. Cells were treated with three kinds of KHC GT-A, -B, and -C of 250 μ g/ml. Data were obtained from three independent trials and presented as mean \pm SD. * p < 0.05 compared to the control group.

that evaluates its effects on the levels of TIMP-1 and MMP-2, important fibrotic mediators.

To apply KHC GT-A for a wide range of liver diseases related to inflammation and fibrosis, this study is aimed at investigating whether KHC GT-A inhibits the production of IL-6, TNF- α , and IL-10 in LPS-stimulated RAW 264.7 cells and α -SMA, collagen1A1, TIMP1, and MMP-2 in LX-2 cells for the first time. In addition, its actions on enhanced IL-6 synthesis are analyzed in LPS-induced LX-2 cells. Furthermore, these experiments are repeated in the samples treated with KHC GT-B and -C, new formulas condensed from KHC GT-A.

IL-6 and TNF- α are crucial pro-inflammatory mediators involved in liver inflammation such as NAFLD and viral hepatitis³⁷) as well as chronic liver

fibrosis. Especially, IL-6 stimulated collagen synthesis in rats³⁸) and higher hepatic TNF- α was observed in more severe liver fibrosis³⁹). On the other hand, IL-10 inflammatory cytokines are recently shown to be related to tissue remodeling, protecting from liver fibrogenesis. Collagen1A1 and α -SMA are predominant markers in fibrosis, mainly expressed in the transition from quiescent HSCs into myofibroblasts¹¹). Early increase of MMP-2 promotes fibrosis and TIMP1 activates anti-apoptosis of HSCs, thus increasing fibrogenic cell survival⁴⁰⁻⁴¹).

As shown in Fig. 2, KHC GT-A of 250 μ g/ml significantly inhibited IL-6 enhanced by LPS in RAW 264.7 cells (p < 0.001), while the level of IL-10 secretion was maintained similar to LPS-induced group after treating KHC GT-A (Fig. 3). Anti-fibrotic activities of

KHCGT-A through the maintenance of IL-10 seem to be associated with significant reduction of TIMP1 in LX-2 cells ($p < 0.05$, Fig. 4). Both KHCGT-B and -C significantly suppressed IL-6, TNF- α , and IL-10 levels activated in LPS-induced RAW 264.7 cells. Interestingly, KHCGT-C composed of only 3 herbs led to the most drastic reduction in the level of pro-inflammatory factors in RAW 264.7 cells and LX-2 cells, both stimulated by LPS treatment. However, KHCGT-C significantly suppressed IL-10 levels, anti-fibrotic mediators, as well as collagen1A1 and MMP-2 mRNA expression. Probably, there are various steps of complicated inflammatory and fibrogenic pathological process, and each formula can intervene different steps.

In conclusion, this study demonstrates that KHCGT shows an inhibitory effect on the expression of pro-inflammatory cytokines such as IL-6, TNF- α , and IL-10 in LPS-stimulated RAW 264.7 cells as well as fibrosis-related genes in LX-2 cells. Among three formulas, KHCGT-C showed the most outstanding efficacy particularly in suppressing inflammatory mediators in LPS-induced RAW 264.7 and LX-2 cells and the expression of collagen1A1 and MMP-2 mRNA in LX-2 cells. Its pharmacological properties might contribute to the development of anti-inflammatory, hepato-protective, and anti-fibrotic agents. However, the molecular mechanism responsible for suppressing inflammatory mediators by KHCGT treatment has not been yet elucidated. Therefore, further investigations are required to clarify molecular signaling pathway of KHCGT related to anti-inflammatory and anti-fibrotic effects in various pathological models.

References

1. Krakauer T. Molecular therapeutic targets in inflammation: cyclooxygenase and NF- κ B. *Current Drug Targets-Inflammation & Allergy*. 2004;3(3):317-324.
2. Tseng CK, Lin CK, Chang HW, Wu YH, Yen FL, Chang FR, et al. Aqueous extract of *Gracilaria tenuistipitata* suppresses LPS-induced NF- κ B and MAPK activation in RAW 264.7 and rat peritoneal macrophages and exerts hepatoprotective effects on carbon tetrachloride-treated rat. *PLoS one*. 2014;9(1):e86557.
3. Kofler S, Nickel T, Michael W. Role of cytokines in cardiovascular diseases: a focus on endothelial responses to inflammation. *Clinical science*. 2005;108(3):205-213.
4. Mosser DM, Edwards JP. Exploring the full spectrum of macrophage activation. *Nature reviews immunology*. 2008;8(12):958-969.
5. Kundu JK, Surh YJ. Inflammation: gearing the journey to cancer. *Mutation Research/Reviews in Mutation Research*. 2008;659(1):15-30.
6. Thompson K, Maltby J, Fallowfield J, McAulay M, Millward-Sadler H, Sheron N. Interleukin-10 expression and function in experimental murine liver inflammation and fibrosis. *Hepatology*. 1998;28(6):1597-1606.
7. Tilg H, Wilmer A, Vogel W, Herold M, Nölchen B, Judmaier G, et al. Serum levels of cytokines in chronic liver diseases. *Gastroenterology*. 1992;103(1):264-274.
8. Ghazwani M, Zhang Y, Gao X, Fan J, Li J, Li S. Anti-fibrotic effect of thymoquinone on hepatic stellate cells. *Phytomedicine*. 2014;21(3):254-260.
9. Yan HJ, Kwon SY, Kim KS, Jeong HS, Kim Y, Chung WS, et al. Anti-fibrotic effects of Kyungheechunggan-tang on activated hepatic stellate cells and rat liver. *Molecular & Cellular Toxicology*. 2014;10(4):411-421.
10. Barbero-Becerra VJ, Giraudi PJ, Chávez-Tapia NC, Uribe M, Tiribelli C, Rosso N. The interplay between hepatic stellate cells and hepatocytes in

- an in vitro model of NASH. *Toxicology in Vitro*. 2015;29(7):1753-1758.
11. Zhang DQ, Sun P, Jin Q, Li X, Zhang Y, Zhang YJ, et al. Resveratrol Regulates Activated Hepatic Stellate Cells by Modulating NF- κ B and the PI3K/Akt Signaling Pathway. *Journal of food science*. 2016;81(1):240-245.
 12. Benyon R, Iredale J. Is liver fibrosis reversible? *Gut*. 2000;46(4):443-446.
 13. Dufour JF, DeLellis R, Kaplan MM. Reversibility of hepatic fibrosis in autoimmune hepatitis. *Annals of internal medicine*. 1997;127(11):981-985.
 14. Friedman SL, Bansal MB. Reversal of hepatic fibrosis—fact or fantasy? *Hepatology*. 2006;43(S1):S82-S88.
 15. Bataller R, Brenner DA. Hepatic stellate cells as a target for the treatment of liver fibrosis. *Seminars in liver disease*. 2001;21(3):437-452.
 16. Cheng K, Mahato RI. Gene modulation for treating liver fibrosis. *Critical Reviews™ in Therapeutic Drug Carrier Systems*. 2007;24(2):93-146.
 17. Davis GL, Albright JE, Cook SF, Rosenberg DM. Projecting future complications of chronic hepatitis C in the United States. *Liver Transplantation*. 2003;9(4):331-338.
 18. Kiemer AK, Müller C, Vollmar AM. Inhibition of LPS-induced nitric oxide and TNF- α production by α -lipoic acid in rat Kupffer cells and in RAW 264.7 murine macrophages. *Immunology and cell biology*. 2002;80(6):550-557.
 19. Brown KE, Broadhurst KA, Mathahs MM, Brunt EM, Schmidt WN. Expression of HSP47, a collagen-specific chaperone, in normal and diseased human liver. *Laboratory investigation*. 2005;85(6):789-797.
 20. Arthur MJ. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. 2000;279(2):G245-G249.
 21. Mormone E, George J, Nieto N. Molecular pathogenesis of hepatic fibrosis and current therapeutic approaches. *Chemico-biological interactions*. 2011;193(3):225-231.
 22. Murphy FR, Issa R, Zhou X, Ratnarajah S, Nagase H, Arthur MJ, et al. Inhibition of apoptosis of activated hepatic stellate cells by tissue inhibitor of metalloproteinase-1 is mediated via effects on matrix metalloproteinase inhibition implications for reversibility of liver fibrosis. *Journal of Biological Chemistry*. 2002;277(13):11069-11076.
 23. Fowell AJ, Collins JE, Duncombe DR, Pickering JA, Rosenberg WM, Benyon RC. Silencing tissue inhibitors of metalloproteinases (TIMPs) with short interfering RNA reveals a role for TIMP-1 in hepatic stellate cell proliferation. *Biochemical and biophysical research communications*. 2011;407(2):277-282.
 24. Han CW, Lee JH. Effects of KHchunggan-tang on the nonalcoholic fatty liver disease in palmitate-induced cellular model. *The Journal of Korean Medicine*. 2010;32(1):109-120.
 25. Hong SH, Seo SH, Lee JH, Choi BT. The aqueous extract from *Artemisia capillaris* Thunb. inhibits lipopolysaccharide-induced inflammatory response through preventing NF- κ B activation in human hepatoma cell line and rat liver. *International journal of molecular medicine*. 2004;13(5):717-720.
 26. Han JM, Kim HG, Choi MK, Lee JS, Lee JS, Wang JH, et al. *Artemisia capillaris* extract protects against bile duct ligation-induced liver fibrosis in rats. *Experimental and Toxicologic Pathology*. 2013;65(6):837-844.
 27. Hong X, Tang H, Wu L, Li L. Protective effects of the *Alisma orientalis* extract on the experimental nonalcoholic fatty liver disease. *Journal of Pharmacy and Pharmacology*. 2006;58(10):1391-1398.
 28. Dong J, Shao W, Yan P, Cai X, Fang L, Zhao

- X, et al. Curcuminolide, a unique sesquiterpenoid with anti-inflammatory properties from *Curcuma wenyujin*. *Bioorganic & medicinal chemistry letters*. 2015;25(2):198-202.
29. Kao ES, Wang CJ, Lin WL, Yin YF, Wang CP, Tseng TH. Anti-inflammatory potential of flavonoid contents from dried fruit of *Crataegus pinnatifida* in vitro and in vivo. *Journal of agricultural and food chemistry*. 2005;53(2):430-436.
30. Ahn M, Park JS, Chae S, Kim S, Moon C, Hyun JW, et al. Hepatoprotective effects of *Lycium chinense* Miller fruit and its constituent betaine in CCl₄-induced hepatic damage in rats. *Acta histochemica*. 2014;116(6):1104-1112.
31. Kim HP, Lee EJ, Kim YC, Kim J, Kim HK, Park JH, et al. Zeaxanthin dipalmitate from *Lycium chinense* fruit reduces experimentally induced hepatic fibrosis in rats. *Biological and Pharmaceutical Bulletin*. 2002;25(3):390-392.
32. Ceccarelli S, Panera N, Mina M, Gnani D, De Stefanis C, Crudele A, et al. LPS-induced TNF- α factor mediates pro-inflammatory and pro-fibrogenic pattern in non-alcoholic fatty liver disease. *Oncotarget*. 2015;6(39):41434-41452.
33. Tsukamoto H, Matsuoka M, French SW. Experimental models of hepatic fibrosis: a review. *Seminars in liver disease*. 1990;10(1):56-65.
34. Hong M, Li S, Tan HY, Wang N, Tsao SW, Feng Y. Current status of herbal medicines in chronic liver disease therapy: the biological effects, molecular targets and future prospects. *International journal of molecular sciences*. 2015;16(12):28705-28745.
35. Lee HJ, Zerlin T, Kim YH, Lee BE, Song HY. Immunomodulation potential of *Artemisia capillaris* extract in rat splenocytes. *International Journal of Phytomedicine*. 2013;5(3):356-361.
36. Han CW, Kwun MJ, Kim KH, Choi JY, Oh SR, Ahn KS, et al. Ethanol extract of *Alismatis Rhizoma* reduces acute lung inflammation by suppressing NF- κ B and activating Nrf2. *Journal of Ethnopharmacology*. 2013;146(1):402-410.
37. Robert S, Gicquel T, Bodin A, Lagente V, Boichot E. Characterization of the MMP/TIMP imbalance and collagen production induced by IL-1 β or TNF- α release from human hepatic stellate cells. *PLoS one*. 2016;11(4):e0153118.
38. Choi I, Kang HS, Yang Y, Pyun KH. IL-6 induces hepatic inflammation and collagen synthesis in vivo. *Clinical & Experimental Immunology*. 1994;95(3):530-535.
39. Tomita K, Tamiya G, Ando S, Ohsumi K, Chiyo T, Mizutani A, et al. Tumour necrosis factor α signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. *Gut*. 2006;55(3):415-424.
40. Zhou X, Hovell CJ, Pawley S, Hutchings MI, Arthur MJ, Iredale JP, et al. Expression of matrix metalloproteinase-2 and -14 persists during early resolution of experimental liver fibrosis and might contribute to fibrolysis. *Liver International*. 2004;24(5):492-501.
41. Bataller R, Brenner DA. Liver fibrosis. *Journal of clinical investigation*. 2005;115(2):209-218.

ORCID

Junghan Bae: <https://orcid.org/0000-0002-3833-0756>

Eungyeong Jang: <https://orcid.org/0000-0002-9992-3942>

Jang-Hoon Lee: <https://orcid.org/0000-0002-4881-7357>