The Herbal Composition Gangjihwan from *Ephedra intermedia*, *Lithospermum erythrorhizon* and *Rheum palmatum* Ameliorates Hepatic Inflammation and Fibrosis in Obese C57BL/6J Mice and HepG2 Cells

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It was demonstrated that Gangjihwan (DF), which is the herbal composition composed of *Ephedra intermedia*, *Lithospermum erythrorhizon*, and *Rheum palmatum*, inhibits obesity and hepatic steatosis in high fat diet (HFD)-fed obese mice. The aim of this study was to determine the effects of DF on visceral obesity, hepatic inflammation and fibrosis and the mechanism of actions involved in this process using *in vivo* and *in vitro* approaches. DF was extracted with water (DF-FW), 30% grain alcohol (DF-GA30), and 70% grain alcohol (DF-GA70). Administration of DF to HFD-fed control mice decreased visceral tissue mass and visceral adipocyte size without adverse effects. Visceral fat mass was decreased by DF-GA30 and DF-GA70, and visceral adipocyte size by all three DF extracts compared with obese control mice. Histological analysis revealed that three kinds of DF extracts reduced toluidine blue-stained mast cells and collagen accumulation in the liver, the extents of which were most eminent in DF-GA70-treated mice. DF-GA70 decreased the mRNA levels of the inflammation (*TNF α* and *VCAM-1*), fibrosis (*α -SMA*), and apoptosis (*caspase 3*) genes, but increasing the anti-apoptosis gene (*Bcl-2*) mRNA levels in the liver of obese control mice. Consistent with the *in vivo* data, GA-70 also altered the expression of inflammation genes (*TNF α* and *MCP-1*) in HepG2 cells. These results indicate that DF not only inhibits visceral obesity, but also ameliorates visceral obesity-induced hepatic inflammation and fibrosis and that this process may be mediated by regulating the hepatic expression of inflammatory and fibrogenic genes.

Key Words: Herbal medicine, Visceral obesity, Hepatic inflammation, Hepatic fibrosis

Nonalcoholic fatty liver disease (NAFLD) is a pathological condition that covers from lipid accumulation in the liver (simple steatosis) to nonalcoholic steatohepatitis (NASH), fibrosis, and irreversible cirrhosis. NAFLD is a main cause of liver disease worldwide, with the prevalence between 10 to 30%. The prevalence of NAFLD has risen drastically in parallel with a rapid increase in obesity (Charlton, 2004; Vuppalanchi and Chalasani, 2009). In Western society, the NAFLD prevalence is estimated to be 20~30%, whereas it rises to 57~74% among obese patients, increasing up to

90% in morbidly obese subjects (Angulo, 2002; Machado et al., 2006; Milic and Stimac, 2012). There is evidence showing that NAFLD prevalence in the Asian countries is also rapidly growing due to the surge in obesity (Park et al., 2006; Fan, 2013; Chan et al., 2013; Koh et al., 2016).

Visceral obesity is particularly associated with NAFLD (Van der Poorten et al., 2008). For example, NAFLD is more common among Asians than among Caucasians, because visceral obesity occurs more frequently among Asians than among Caucasians with similar body mass index (Petersen

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et al., 2006; Schwimmer et al., 2006). It is known that the area and amount of visceral adipose tissue are deeply associated with the severity of hepatic inflammation and fibrosis in NAFLD patients (Van der Poorten et al., 2008; Yu et al., 2015).

Despite the large numbers of drugs used to treat NAFLD and NASH, there is no current established therapy for NAFLD and NASH other than lifestyle modifications. Because NAFLD occurs primarily in obese patients, weight reduction through lifestyle intervention, bariatric surgery, and drugs is the first-line treatment for all NAFLD patients. Current potential medications for NAFLD include insulin sensitizers such as metformin and thiazolidinediones, lipid-lowering drugs, antioxidants, glucagon-like peptide 1 analogues, and omega-3 fatty acids. However, since long-term intake of these drugs increases the risk of cancer, vascular disease, and depression, there is an urgent need for effective and safe therapeutic prescriptions to overcome NAFLD. Recent studies show that medicinal plants inhibit obesity and improve metabolic disorders including hyperlipidemia, insulin resistance, and NAFLD (Jeong et al., 2008; Shin et al., 2010; Roh et al., 2015; Yoon et al., 2017). Gangjihwan (DF), which is the herbal composition composed of three medicinal plants *Ephedra intermedia* Schrenk et C. A. Mayer, *Lithospermum erythrorhizon* Siebold et Zuccarini, and *Rheum palmatum* L., has been used to treat obesity in local clinics in Korea, although the mechanism of its action remains poorly understood.

Therefore, the effects of DF on visceral obesity and visceral obesity-induced hepatic inflammation and fibrosis were examined in high fat diet (HFD)-induced obese C57BL/6J mice and HepG2 cells, and investigated the histological and molecular events involved in this process. The present results demonstrate that DF inhibits visceral obesity, infiltration of inflammatory cells into the liver, and hepatic collagen accumulation and that this process may be mediated by regulating the hepatic expression of inflammation and fibrosis genes. Thus, DF may attenuate visceral obesity-induced hepatic fibroinflammation.

DF was prepared from food-grade extracts of the three medicinal herbs (40% *Ephedra intermedia*, 40% *Lithospermum erythrorhizon*, and 20% *Rheum palmatum*; ex-

pressed as % dry weight of DF) (Hwalim, Busan, Korea). The proportions used in this study are same as those used to treat patients in local clinics of Korea. Briefly, dried herbs were individually powdered and extracted with water (DF-FW), 30% grain alcohol (DF-GA30), and 70% grain alcohol (DF-GA70). The extracts were freeze-dried under vacuum and mixed for DF production. The extraction yields were 22.7%, 26.33%, and 22.9% for DF-FW, DF-GA30, and DF-GA70, respectively.

Eight-week-old male wild-type C57BL/6J mice (n=7/group) were housed and bred under pathogen-free conditions with a standard 12-h light/dark cycle. Prior to the administration of special diets, mice were fed standard rodent chow and water *ad libitum*. Mice were randomly divided into five groups: Mice fed a low fat diet (LFD; 10 kcal% fat, Research Diets, New Brunswick, NJ, USA) and received water by oral gavage for 8 weeks (Normal). The second group fed a HFD (45 kcal% fat, Research Diets) and received water by oral gavage (Control). The third group fed a HFD and received DF-FW (250 mg/kg BW) by oral gavage (DF-FW). The fourth group fed a HFD and received DF-GA30 (250 mg/kg BW) by oral gavage (DF-GA30). The fifth group received a HFD and received DF-GA70 (250 mg/kg BW) by oral gavage (DF-GA70). After an 8 h fast on the last day of the study, the animals were sacrificed by cervical dislocation. Liver tissues were removed, weighed, snap-frozen in liquid nitrogen and stored at -80 °C until use. Portions of the Liver and visceral adipose tissues were prepared for histology. All animal experiments were approved by the Institutional Animal Care and Use Committee of Donggeui University (permit number: R2015-009) and followed National Research Council Guidelines.

Tissue specimens were fixed in 10% phosphate-buffered formalin for 1 day and embedded in paraffin. To examine the hepatic inflammation, fibrosis, and apoptosis, liver tissue sections (5 µm) were cut and stained with toluidine blue, Masson's trichrome, and 4',6-diamidino-2-phenylindole (DAPI), respectively. To measure the visceral adipocyte size, epididymal adipose tissue (5 µm) was stained with hematoxylin and eosin. Stained preparations were analyzed using an image analysis system (Media cybernetics, Bethesda, MD).

HepG2 cells, a well-differentiated human hepatoblastoma cell line, were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Cells were cultured for 2 days until reaching 70~80% confluency. Cells were treated with various concentrations of three DF extracts in the presence of a mixture of oleic acid and palmitoleic acid for 2 days. Oleic acid and palmitoleic acid were dissolved in ethanol and isopropanol at concentrations of 0.8 and 0.4 mM, respectively. Cell viability was detected by 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide disodium salt

(XTT) assay using a Cell Proliferation Kit II (Roche, Basel, Switzerland).

Total cellular RNA from mouse liver tissue and HepG2 cells was prepared using Trizol reagent (Gibco-BRL, Grand Island, NY, USA). Total cellular RNA (2 µg) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase to generate an antisense cDNA template. The genes of interest were amplified from the synthesized cDNA using *AccuPower*[®] *GreenStar*[™] qPCR PreMix (Bioneer, Deajeon, Korea) on an *Excycler*[™] 96 Real Time Quantitative Thermal Block machine (Bioneer). The PCR primers used

Table 1. Sequences of primers used for quantitative real-time PCR assays

Genes	Gene bank	Primer sequences
<u>Human</u>		
MCP-1	NM_002982.3	Forward : 5'- CACTCACTCCACAACCCAAGA -3' Reverse : 5'- CAAAGACCCTCAAAACATCCC -3'
TNF α	NM_000594.3	Forward : 5'- AGCCCATGTTGTAGCAAACC -3' Reverse : 5'- GGAAGACCCCTCCCAGATAG -3'
<u>Mouse</u>		
α -SMA	NM_007392.3	Forward : 5'- CTGGAGAAGAGCTACGAACTGC -3' Reverse : 5'- CTGATCCACATCTGCTGGAAGG -3'
Bcl-2	NM_009741.5	Forward : 5'- TCGCTACCGTCGTGACTTC -3' Reverse : 5'- AAACAGAGGTCGCATGCTG -3'
Caspase 3	NM_009810.3	Forward : 5'- ACGCAGCCAACCTCAGAGA -3' Reverse : 5'- ATGAACCACGACCCGTCCT -3'
Caspase 8	NM_001277926.1	Forward : 5'- TGCTTGGACTACATCCCACAC -3' Reverse : 5'- CCAACTCGCTCACTTCTTCTG -3'
CD68	NM_001291058.1	Forward : 5'- AACAGGACCTACATCAGAGC -3' Reverse : 5'- CTGTAGCCTTAGAGAGAGCA -3'
Collagen α 1	NM_007742.4	Forward : 5'- GCCCGAACCCCAAGGAAAAGAAGC -3' Reverse : 5'- CTGGGAGGCTCGGTGGACATTAG -3'
ICAM-1	NM_010493.3	Forward : 5'- AGCTAGCGGACCAGATCC -3' Reverse : 5'- ATACAGCACGTGCAGTTCC -3'
MCP-1	NM_011333.3	Forward : 5'- TGATCCCAATGAGTAGGCTGGAG -3' Reverse : 5'- ATGTCTGGACCCATTCTTCTTG -3'
TGF β 1	NM_011577.2	Forward : 5'- ACCGCAACAACGCCATCTAT -3' Reverse : 5'- GTAACGCCAGGAATTGTTGC -3'
TNF α	NM_001278601.1	Forward : 5'- GGCAGGTCTACTTTGGAGTCATTGC -3' Reverse : 5'- ACATTCGAGGCTCCAGTGAATTCGG -3'
VCAM-1	NM_011693.3	Forward : 5'- GGAAATGCCACCCTCACCTTA -3' Reverse : 5'- GATTTGGCCCCCTCATTCCTT -3'

for gene expression analysis are shown in Table 1. PCR was performed using the following conditions: denaturing at 95°C for 5 min followed by 50 cycles of 95°C for 10 s, 60°C for 40 s, and 72°C for 10 s. PCR products were calculated as copies per µl using a standard curve, and the relative expression levels were calculated as the ratio of target gene cDNA to β-actin cDNA.

Values are expressed as mean ± standard deviation (SD). Statistical analysis was performed using analysis of variance followed by Turkey's post-hoc tests. Statistical significance was defined as a *P*-value <0.05.

The prevalence of NAFLD has risen rapidly in parallel with a dramatic increase in obesity (Charlton, 2004; Vuppalanchi and Chalasani, 2009). Visceral obesity is particularly associated with the NAFLD development including hepatic inflammation and fibrosis (Van der Poorten et al., 2008; Yu et al., 2015). To determine the effect of DF on visceral obesity, HFD-fed obese mice were treated with DF-FW, DF-GA30, and DF-GA70 for 8 weeks. HFD-fed control mice had much higher visceral fat mass and visceral adipocyte size compared with LFD-fed normal mice. However, treatment of obese mice with DF-GA30 and DF-GA70 reduced visceral adipose tissue weight compared with control mice (Fig. 1A). Visceral adipocyte size was also decreased by all three DF extracts (Fig. 1B), suggesting that DF may inhibit visceral obesity and visceral adipocyte hypertrophy. It is known that larger adipocytes in visceral adipose tissue secrete free fatty acids and inflammatory cytokines such as monocyte chemoattractant protein 1 (MCP-1) and tumor necrosis factor α (TNFα) compared with smaller adipocytes, leading to metabolic syndromes including insulin resistance, type 2 diabetes, NAFLD, and cardiovascular disease (Xu et al., 2003; Curat et al., 2004; Despres and Lemieux, 2006; Jeong and Yoon, 2009). Thus, DF can regulate obesity and adipocyte hypertrophy, and might be a novel therapeutic approach to treat obesity and related metabolic diseases.

Visceral obesity promotes hepatic inflammation and fibrosis in rodents and humans (Cai et al., 2005; Canello et al., 2006; Mulder et al., 2016; Van der Poorten et al., 2008; Yu et al., 2015). To determine whether DF regulates hepatic inflammation, liver sections were stained with toluidine blue to detect mast cells. Control mice showed increased toluidine

blue-stained mast cells in the liver compared with normal mice (Fig. 2A). Consistent with the changes in visceral fat mass and adipocyte size, three kinds of DF extracts reduced mast cells in the liver. Of the three DF extracts, DF-GA70 was most effective in decreasing the hepatic inflammation in obese mice. Liver inflammation is increased by the expression in hepatocytes of inflammatory genes, such as *MCP-1*, *TNFα*, *CD68*, intercellular adhesion molecule-1 (*ICAM-1*), and vascular cell adhesion molecule-1 (*VCAM-1*) (Cai et al., 2005; Chatzigeorgiou et al., 2014; Tilg and

Fig. 1. Regulation of visceral fat weight and visceral adipocyte size by DF in HFD-fed obese mice. Adult male mice were fed an LFD (normal), an HFD (control), or the HFD supplemented with DF-FW, DF-GA30, and DF-GA70 for 8 weeks. (A) Modulation of visceral fat weight by DF. (B) Regulation of visceral adipocyte size by DF. The size of adipocytes in a fixed area (1,000,000 µm²) was measured. #*P*<0.05 compared to the normal group, **P*<0.05 compared to the control group.

Moschen, 2008). Expression levels of these inflammatory genes were increased in HFD-fed control mice compared

with LFD-fed normal mice (Fig. 2B). In contrast, DF-GA70 treatment decreased *TNF α* and *VCAM-1* mRNA levels in

Fig. 2. Inhibition of hepatic inflammation by DF in HFD-fed obese mice. Adult male mice were fed an LFD (normal), an HFD (control), or the HFD supplemented with DF-FW, DF-GA30, and DF-GA70 for 8 weeks. (A) Representative toluidine blue-stained sections of liver tissues (original magnification $\times 400$). (B) Expression of genes involved in inflammation. All values are expressed as the mean $\pm$ SD. [#] $P < 0.05$ compared to the normal group, ^{*} $P < 0.05$ compared to the control group.

control mice. These results indicate that DF may inhibit visceral obesity-induced hepatic inflammation by downregulation of TNF α and VCAM-1.

Hepatic fibrosis is characterized by the excessive accumulation of extracellular matrix (ECM) proteins including collagen, which occurs in most chronic liver disease. Pathogenesis of hepatic fibrosis includes hepatic stellate cell (HSC) activation, and expression and accumulation of ECM proteins. Cytokines such as transforming growth factor β 1 (TGF β 1) induce fibrosis by arresting activated HSC that produce ECM proteins (Saile et al., 2001). To determine whether DF regulates hepatic fibrosis, liver sections were

stained Masson's trichrome. Control mice showed Masson's trichrome-stained hepatic collagen accumulation in the liver compared with normal mice (Fig. 3A). However, hepatic collagen levels were reduced in mice treated with all three kinds of DF extracts, and DF-GA70 treatment almost completely cleared hepatic collagen in obese mice. Hepatic expression of TGF β 1, α -SMA, and collagen α 1 is closely linked to hepatic fibrosis (Bataller and Brenner, 2005). To evaluate whether the inhibitory effects of DF on hepatic fibrosis in obese mice were associated with alterations of the expression of fibrosis genes such as *TGF β 1*, α smooth muscle actin (α -SMA), and *collagen α 1*. *Alpha-SMA* mRNA levels were

Fig. 3. Inhibition of hepatic fibrosis by DF in HFD-fed obese mice. Adult male mice were fed an LFD (normal), an HFD (control), or the HFD supplemented with DF-FW, DF-GA30, and DF-GA70 for 8 weeks. (A) Representative Masson's trichrome-stained sections of liver tissues (original magnification $\times 100$). (B) Expression of genes involved in fibrosis. All values are expressed as the mean $\pm$ SD. [#] $P < 0.05$ compared to the normal group, ^{*} $P < 0.05$ compared to the control group.

decreased by all three DF extracts (Fig. 3B). These results suggest that DF inhibits hepatic inflammation by upregulation of α -SMA in HFD-fed obese mice.

NAFLD is accompanied by hepatocyte apoptosis (Cao et al., 2016). Apoptotic bodies stimulate hepatic inflammation and fibrosis, and the magnitude of apoptosis correlates with stage of fibrosis in patients with NASH (Feldstein et al., 2003). Inhibition of hepatocyte apoptosis with a caspase inhibitor reduced hepatic injury and fibrosis in mice with liver injury (Canbay et al., 2004). In the present study, DAPI-stained liver sections revealed that HFD-fed control mice did not exhibit increased hepatocyte apoptosis compared

with normal mice, suggesting that mild hepatic inflammation and fibrosis following HFD feeding may be due to the low level of apoptotic hepatocytes in this mouse model (Fig. 4A). Expectedly, hepatocyte apoptosis was not affected by DF treatment. Although, analysis of the expression of apoptosis (*caspase 3* and *caspase 8*) and anti-apoptosis (B cell lymphoma 2 (*Bcl-2*)) genes showed that HFD feeding increased *caspase 3* mRNA levels with decreasing *Bcl-2* mRNA levels in the liver (Fig. 4B). In contrast, DF-GA30 and DF-GA70 decreased *caspase 3* mRNA levels, whereas DF-FW and DF-GA70 increased *Bcl-2* mRNA levels in the liver. These results indicate that DF regulates apoptotic

Fig. 4. Modulation of hepatocyte apoptosis by DF in HFD-fed obese mice. Adult male mice were fed an LFD (normal), an HFD (control), or the HFD supplemented with DF-FW, DF-GA30, and DF-GA70 for 8 weeks. (A) Representative DAPI-stained sections of liver tissues (original magnification $\times 100$). (B) Expression of genes involved in apoptosis. All values are expressed as the mean $\pm$ SD. [#] $P < 0.05$ compared to the normal group, ^{*} $P < 0.05$ compared to the control group.

gene expression although hepatocyte apoptosis was not observed in the histological analysis.

It is known that an NAFLD cell model is induced by an overload of free fatty acids (Gomez-Lechon et al., 2007; Liang et al., 2015). The previous study demonstrated that treatment of HepG2 cells with excess free fatty acids induced steatosis and regulated the mRNA expression of lipid metabolism genes (Yoon et al., 2017). Thus, the expression of genes involved in inflammation was examined in HepG2 cells treated with a mixture of free fatty acids such as oleic acid and palmitoleic acid. Free fatty acids increased the mRNA levels of *TNF α* and *MCP-1* (Fig. 5A). However, mRNA levels of these genes were decreased by DF-GA70.

In addition, the XTT assays showed that the viability of HepG2 cells was not affected by DFs at all concentrations used in this study (Fig. 5B). These results suggest that DF inhibits the expression of inflammatory genes in HepG2 cells, supporting an inhibitory role of DF on hepatic inflammation *in vivo*.

In conclusion, these results demonstrate that DF, a polyherbal drug composed of *Ephedra intermedia*, *Lithospermum erythrorhizon*, and *Rheum palmatum*, inhibits visceral obesity and ameliorates hepatic fibroinflammation by regulating the expression of inflammation and fibrosis genes in animal and cell models of human NAFLD. In addition, these inhibitory effects were most effective in mice treated with DF-GA70.

Fig. 5. Effects of DF on mRNA expression of inflammation genes in HepG2 cells. Cells were treated with various concentrations of DF-FW, DF-GA30, and DF-GA70 in the presence of fatty acids. Control cells were treated with fatty acids only. (A) Expression of inflammation genes. (B) Effects of DF on HepG2 cell viability by XTT assay. All values are expressed as the mean $\pm$ SD. * $P < 0.05$ compared to the control group.

These results suggest that DF may be an effective drug for preventing and treating obesity and NAFLD. Further studies will be necessary to establish the experimental conditions (e.g. longer duration of DF treatment and higher doses of DF) equivalent to human hepatic inflammation, fibrosis, and apoptosis, and to identify active compounds of each individual herbs for their effects on obesity and NAFLD.

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CONFLICT OF INTEREST

None declared.

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